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IL-36R SIGNALING AND GUT IMMUNITY

by

VU NGO

Under the Direction of Timothy L. Denning, PhD

ABSTRACT

Inflammatory bowel disease (IBD) is a chronic, relapsing inflammatory disorder of the gastrointestinal tract. Cytokines have a critical role in the pathogenesis of IBD, in which they control multiple aspects of the inflammatory response. One such cytokine, Interleukin (IL-36) is overexpressed in both experimental and human IBD and may play both pathogenic and protective roles, depending on the context. The IL-36 signaling pathway induces IL-22, a cytokine that is critical in repair and regeneration of the gut epithelium during inflammation. However, the mechanism by which IL-36 induces IL-22 and promotes barrier repair is incompletely understood.

We observed a cytokine network, in which, IL-36 γ /IL-36R signaling is an important upstream driver of IL-22 and antimicrobial peptide (AMP) production to protect the host against acute intestinal damage. We have shown that IL-36 γ is a potent inducer of IL-23. IL-36 γ -induced

IL-23 requires Notch2-dependent dendritic cells (DCs). The intracellular signaling cascade linking IL-36 receptor (IL-36R) to IL-23 production by DCs involved MyD88 and the NF- κ B subunits. Consistent with *in vitro* observations, IL-36R^{-/-} mice exhibited dramatically reduced IL-23, IL-22, and AMP levels, and consequently failed to recover from acute intestinal damage. Interestingly, impaired recovery of mice deficient in IL-36R was rescued by treatment with exogenous IL-23. We also found that IL-36R^{-/-} mice are defective of host protection during enteropathogenic bacterial infection. The IL-36 signaling cascade is essential in both early and late phases of immune defense against *C. rodentium* infection. During the early stage of infection, IL-36 induced the production of IL-22 by innate lymphoid cells 3 (ILC3s) via IL-23; and during the late phase, IL-36 stimulated the production of IL-22 from T-cells via IL-6. Intriguingly, in the experimental chronic colitis, IL-36R^{-/-} mice display reduced disease severity and are associated with decrease production of inflammatory cytokines and enhances expression of Foxp3 regulatory T cells. Collectively, these findings highlight context-dependent, protective and pathogenic contribution of the IL-36R signaling pathway and the potential for optimally manipulating this pathway for better treatment of intestinal inflammation.

INDEX WORDS: Inflammatory bowel diseases, Intestinal bacterial infection, Interleukin, Acute intestinal inflammation, Chronic intestinal inflammation

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by

VU NGO

A Dissertation Submitted in Partial Fulfillment of the Requirements for the Degree of

Doctor of Philosophy

in the College of Arts and Sciences

Georgia State University

2020

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2020

IL-36R SIGNALING AND GUT IMMUNITY

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May 2020

DEDICATION

I am dedicating this dissertation to two beloved people who have meant and continue to mean so much to me. First and foremost, to my father, Thinh, whose love for me knows no bounds, and for all his sacrifice, he made and continue to make throughout my life. Thank you so much.

Next, my fiancée, Kim Ngan, for her unwavering supports and infinite patience with me through my education; and always there to feed me and cheer me up when my experiments fail.

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LIST OF ABBREVIATIONS

1. IBD	Inflammatory bowel disease
2. UC	Ulcerative colitis
3. DCs	Dendritic cells
4. IEC	Intestinal epithelial cells
5. IL-36R	IL-36 receptor
6. IL-36 α	Interleukin-36 α
7. IL-36 β	Interleukin-36 β
8. IL-36 γ	Interleukin-36 γ
9. IL-23	Interleukin-23
10. IL-22	Interleukin-22
11. IL-6	Interleukin-6
12. EGFs	Epidermal growth factors
13. T-reg	regulatory T-cell
14. NE	Neutrophil Elastase
15. IL1RL2	IL1-R-related protein 2
16. IL1RAcP	IL-1 receptor accessory protein
17. NF κ B	nuclear factor kappa B
18. H&E staining	Hematoxylin/Eosin staining
19. rIL-23	Recombinant Interleukin-23 protein
20. AMPs	Anti-microbial peptides
21. rIL-6	Recombinant Interleukin-6 protein
22. ILC3	Innate lymphoid cells 3
23. NK cells	Natural Killer cells
24. SFB	Segmented filamentous bacteria
25. DSS	Dextran sodium sulfate
26. PBS-T	Phosphate buffered saline + Tween-20
27. BMDCs	Bone marrow-derived dendritic cells
28. FBS	Fetal bovine serum
29. ChIP	Chromatin Immunoprecipitation
30. ELISA	Enzyme-linked immunosorbent assay
31. BMDMs	Bone marrow-derived macrophages
32. MDCs	human monocytes derived dendritic cells
33. GM-CSF	Granulocyte macrophage colony-stimulating factor
34. WT mice	Wild-type mice

1 CHAPTER 1: BRIEF LITERATURE REVIEW

1.1 Inflammatory bowel disease (IBD)

Inflammatory bowel diseases (IBD), comprised of ulcerative colitis (UC) and Crohn's disease, are chronic relapsing disorder affecting the gastrointestinal tract. Affecting approximately 1.5 million Americans, two million European and several hundred thousand more world-wide, IBD are global disorders with high rates of occurrence in developed countries [1-5]. However, since 1990 the rate of incidence of IBD in Western countries has started to decline and stabilize, while the incidence rate in newly industrialized countries in South America, Africa, and Asia is accelerating [6].

Both UC and Crohn's disease are typified by non-infectious chronic inflammation of the digestive tract. The inflammation in UC is characterized by continuous inflammation in the large intestine and limited to the mucosal and submucosal layers. In contrast, irregular inflammation involving all segments of the gastrointestinal tract that occurs anywhere from the oral cavity to the anus is typically an indicator of CD [2, 7, 8]. The symptoms of IBD can range from mild to severe, depending on the stage of the disease and the portion of the gastrointestinal tract affected. Symptoms often include abdominal pain, abnormal frequency of bowel movement, diarrhea, fever, weight loss, and loss of appetite [9, 10]. Although IBD does not affect the patients' life expectancy, it has a substantial impact on their quality of life with IBD patients usually experiencing fatigue during remission and flareup [10]. Moreover, the destructive nature of the disease can lead to complications such as stenoses, abscesses, fistulas, extraintestinal manifestations, and colitis-associated cancer often observed in IBD patients [2, 3, 7, 8].

1.1.1 Pathogenesis of IBD

The etiology of IBD remains unclear; however, it is hypothesized that these disorders are the result of irregular immune responses to microbiota in genetically vulnerable individuals [2, 8]. Studies have shown that environment and genetic factors play a critical role. Gene polymorphisms in loci coding molecules that affect chemokines, cytokine signaling, antimicrobial peptides, and molecules involved in the sensing of immune cells have been revealed to increase the prospect of developing IBD [7, 11, 12]. In addition, environmental factors such as, stress, smoking, and prescribed antibiotics all increase the risk of IBD [13-15]. However, none of the predisposing components mentioned above can act alone to trigger IBD, instead the combination of genetics, environmental factors, and host-microbe interaction appears to prompt IBD-initiating events. This leads to impaired intestinal barrier function and eventually results in the translocation of microorganisms from the lumen to the bowel wall. Consequently, innate immune cells respond to foreign microorganisms and lead to the activation of immune cells and the production of cytokines/chemokines. That process in turn impair gut homeostasis and recruit additional immune cells and causes the activation of adaptive immunity [11-13, 16-20]. Mediators including cytokines and chemokines produced by activated immune cells can then induce epithelial damage, impairment of barrier function and dysbiosis and perpetuate gut inflammation [2, 8, 21].

1.1.2 Cytokines in IBD

Cytokine responses orchestrate the initiation, progression, and resolution of the diseases and are thought to be critical elements in IBD [2, 8]. Upon secretion, these cytokines trigger the differentiation of mucosal naïve T-cells to effector T cells or regulatory T-cell. Different lineages of effector T-cells are crucial for host protection against foreign microbes. However, the imbalance of effector T cells and regulatory T cells can result in chronic IBD. As a result, the immune system

has several mechanisms to control these effector T cells, of which the CD4⁺ regulatory T-cells (T_{reg}) are the most important. This balance between effectors and T-reg is essential for the safeguarding of intestinal homeostasis. Previous studies have indicated that IBD patients can either exhibit T_H1 mediated immune response, which is characterized by up-regulation of TNF, IFN- γ , IL-12 mediated immune response or characterized by increase production of T_H2 related cytokines (IL-4, IL-13) [22-25]. Figures below adapted from Neurath, M. *et al.*, Cytokines in inflammatory bowel disease. *Nat Rev Immunol* 14. illustrated the key cytokines in IBDs [26-29]

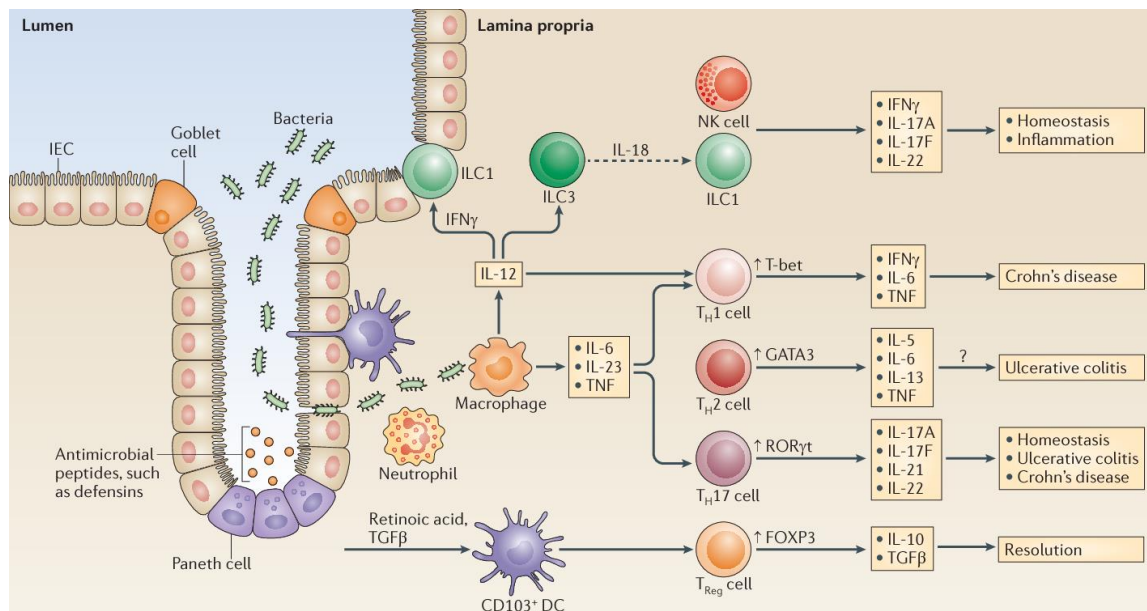


Figure 1.1 Key cytokine in IBDs. From Neurath, M. Cytokines in inflammatory bowel disease. *Nat Rev Immunol* **14**, 329–342 (2014)

1.2 IL-36 Cytokines

The IL-36 cytokines were discovered almost twenty-years ago through genome screening and identified due to their structural homolog to IL-1 [26, 30, 31]. IL-36 cytokine subfamily comprises of four members and originally named as IL-1F6, IL1F8, IL1F9 and IL-1F5 for belong to IL-1 superfamily, but later re-named to IL-36 α , IL-36 β , IL-36 γ and IL-36Ra. The encoding

gene for IL-36 was found to be clustered on chromosome two and subsequently cloned in 2001 [32, 33]. While IL-36 α , IL-36 β and IL-36 γ function as IL-36 receptor agonists, IL-36Ra (IL-36 receptor antagonist) prevents the activation of IL-36R signaling. All IL-36 cytokines ligands bind to the IL-36 receptor complex (IL-36R), which is comprised of the IL1-R-related protein 2 (IL-1RL2) and a co-receptor subunit IL-1 receptor accessory protein (IL1RAcP) [34, 35]. Through a combination of in vitro/in vivo characterizations and structural studies, the functions of IL-36 has begun to unveil over the last 20 years. Depending on the location of expression, phases, and context of the diseases, IL-36 cytokines can either favor inflammation or promote the resolution of inflammation. IL-36 was best comprehensively studied and explored in the context of skin inflammation, such as psoriasis [35, 36]. Over the past decades, the pathological and protective effects of IL-36 cytokines have been expanded to a range of inflammatory diseases including systemic lupus erythematosus, psoriatic, inflammatory bowel diseases [37-47]. In addition, IL-36 cytokines have also been found to respond to inflammatory signals induced bacterial and viruses, indicating their role in anti-bacterial and anti-virus infection [34, 35, 48-53].

1.2.1 Secretion of IL-36

Expression of IL-36 cytokines is observed at low level across different organs including skin, intestines, lung, and brain. During inflammation IL-36 mainly expressed by keratinocytes, epithelial cells, inflammatory monocytes/macrophages. *In vitro* studies demonstrated that Ly6C⁺ monocytes or bone marrow derived macrophages responses to toll-like receptor ligands including LPS, flagellin, CpG, and Poly I:C or inflammatory cytokines (IL-1 β , IL-18) to induces robust secretion of IL-36 γ [32, 34, 48]. In the skin, epidermal growth factors (EGFs) have been identified as triggers for IL-36 cytokine production in the skin during psoriasis. In several models of asthma, lung epithelial cells have been reported to produce IL-36 agonist ligands in responses to

Rhinovirus stimulation. T lymphocytes have been reported to express the IL-36 agonists under influences of fibroblast growth factors (FGFs). In addition, IL-36 α is upregulated in synovium-infiltrated plasma cells of rheumatoid arthritis patients. IL-36 cytokines are also found to be elevated in the lung in response to various bacterial/ viral infections [35, 37, 38, 48, 52, 53]. In the intestine, IL-36 α and IL-36 γ were highly upregulated in multiple models of murine intestinal inflammation as well as in IBDs patients [43, 45-47, 54-56]. In cultured human KCs, IL-36 cytokines can also induce the expression of themselves in an autocrine loop, emphasizing its potent proinflammatory functions [57]. Like its ligands, the IL-36R complex is also widely expressed at low level and does not seem to be upregulated unless there are pathological conditions present. Collectively, these finding suggest that IL-36 cytokines are produced in response to the diseases in a stimuli-dependent fashion; however, the regulatory mechanism triggering the production of IL-36 cytokines remains unknown.

The secretion mechanisms of IL-36 cytokines are not well-known. All IL-36 agonist ligands do have signal sequences or caspases cleavage site; hence, they are release from cells by an endoplasmic reticulum and Golgi independent secretion pathway. Studies established that IL-36 α and IL-36 γ produced by macrophages are accumulated intracellularly and is release in response to ATP stimulation. Kovach et al., demonstrated that IL-36 γ secreted by lung alveolar macrophages within microparticle in responses to bacterial infection [50, 58]. In addition, a recent study showed influenza virus induced capase-1 activation and led to the induce of IL-36 α , however, caspase-3 and caspase-7 are required for alveolar epithelial cells to release IL-36 α [51, 53, 59-61].

1.2.2 Processing of IL-36

Similar with other cytokines in IL-1 superfamily, all IL-36 ligands are secreted in inactive forms (pro-IL-36) and have little or no bioactivity. This low activity could be ascribed to the

absence of proteolytic processing that produces the active forms. All IL-36 agonists must be undergoing N-terminal cleave process by specific proteases to become activates their full biological potentials. Upon cleaved of nine amino acids in the N-terminal portion from the A-X-Asp motif, IL-36 agonists greatly increase their affinity to bind to IL-36R complex [62]. Neutrophils proteases have been recognized as the main regulators of all the IL-36 family. Neutrophil Elastase (NE) is the enzyme responsible for enhancing IL-36Ra activity. Neutrophil-derived proteases, cathepsin G, and elastase activate IL-36 α ; cathepsin G activates IL-36 β while IL-36 γ is activate by proteinase-3 and proteases elastase (Figure 1.1) [34, 59, 63, 64].

1.2.3 IL-36 Signaling

IL-36 ligands and IL-36R are expressed in wide range to tissues including gut, skin, lung, renal and cervical tissue. Once secretion, all IL-36 agonists ligands first bind to IL-1-R-related protein 2 (IL-1RL2), resulted in the recruitment of IL-1 receptor accessory protein (IL1RAcP) to form a functional IL-36R complex. Upon interacting with their receptor, IL-36 agonist triggers intracellular cascades leading to the activation of mitogen-activated protein kinase and nuclear factor kappa B (NF κ B) to induces various immune response [54, 56, 62]. IL-36Ra and IL-38 acts as an antagonist as they bind to IL1RL2 and inhibiting the recruitment of IL-1RAcP and prevent the creation of a heterodimeric IL-36R complex (Figure 1.1) [34, 65].

1.2.4 IL-36 cytokines and their effects on immune cells

IL-36 cytokines play a substantial role in shaping host immunity by exerting their influence on both the innate and adaptive arms of the immune system. Studies have shown that IL-36 has effect on dendritic cells, macrophages, and fibroblasts. Both murine bone marrow-derived dendritic cells (BMDCs) and human monocytes derived dendritic cells (MDCs) express a higher level of IL-36R and response to the stimulation with IL-36 ligands by producing a wide range of

pro-inflammatory cytokines including IL-2, IL-23, IL-6, IL-12, GM-CSF, and TNF- α . These cytokines then facilitate the differentiation of naïve CD4⁺ T cells into T_H1, T_H17/T_H22 effector T cells. Furthermore, IL-36 can also govern the recruitment of innate immune cells by inducing the production of chemo-attractants including CXCL1, CXCL2, CXCL3, and CXCL5 (Figure 1.1) [36, 37, 40, 48, 66, 67].

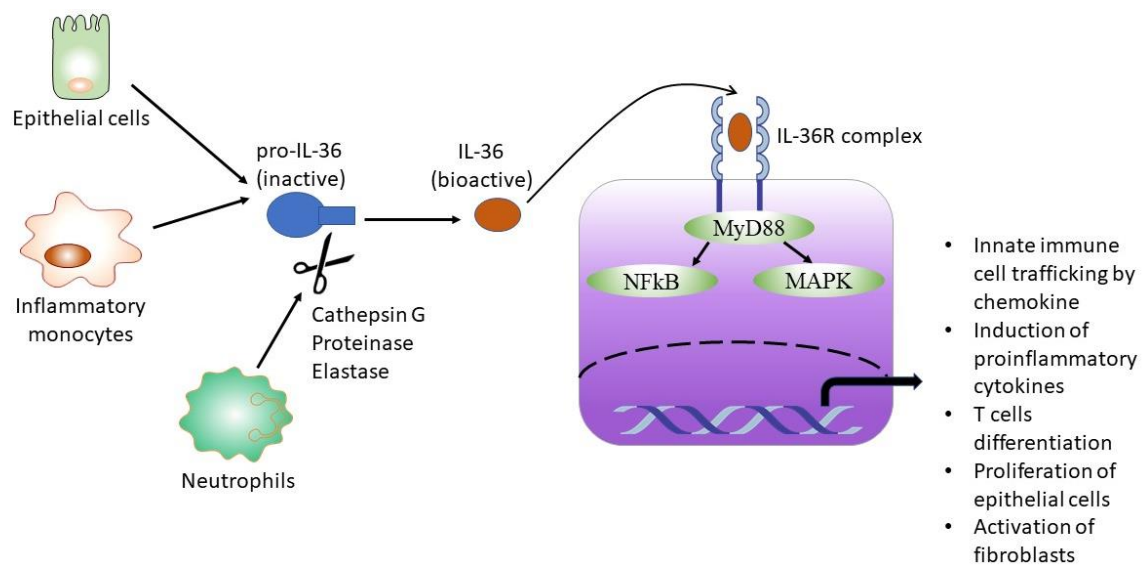


Figure 1.2 Regulation and Functions of IL-36

2 CHAPTER 2: A CYTOKINE NETWORK INVOLVING IL-36 γ , IL-23, AND IL-22 PROMOTES ANTIMICROBIAL DEFENSE AND RECOVERY FROM INTESTINAL BARRIER DAMAGE

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2.1 Abstract

The gut epithelium acts to separate host immune cells from unrestricted interactions with the microbiota and other environmental stimuli. In response to epithelial damage or dysfunction, immune cells are activated to produce interleukin (IL)-22, which is involved in repair and protection of barrier surfaces. However, the specific pathways leading to IL-22 and associated anti-microbial peptide (AMP) production in response to intestinal tissue damage remain incompletely understood. Here we define a critical IL-36/IL-23/IL-22 cytokine network that is instrumental for AMP production and host defense. Using a murine model of intestinal damage and repair, we show that IL-36 γ is a potent inducer of IL-23 both *in vitro* and *in vivo*. IL-36 γ -induced IL-23 was dependent upon Notch2-dependent (CD11b⁺CD103⁺) DCs, but not Batf3-dependent (CD11b⁻CD103⁺) DCs or CSFR1-dependent macrophages. The intracellular signaling cascade linking IL-36R to IL-23 production by DCs involved MyD88 and the NF- κ B subunits c-Rel and p50. Consistent with *in vitro* observations, IL-36R- and IL-36 γ -deficient mice exhibited dramatically reduced IL-23, IL-22 and AMP levels and consequently failed to recover from acute intestinal damage. Interestingly, impaired recovery of mice deficient in IL-36R or IL-36 γ could be rescued by treatment with exogenous IL-23. This recovery was accompanied by a restoration of IL-22 and AMP expression in the colon. Collectively, these data define a cytokine network

involving IL-36 γ , IL-23, and IL-22, that is activated in response to intestinal barrier damage and involved in providing critical host defense.

2.2 Significance statement

Cytokines are produced in response to microbial threat and aid in the recruitment and activation of immune cells in order to protect the host. Using complementary *in vitro* and *in vivo* approaches, we have defined a cytokine network involving IL-36 γ , IL-23, and IL-22 that is induced following intestinal damage and critical for antimicrobial activity, tissue repair and host survival. Our data identify IL-36 γ /IL-36R signaling as a central upstream driver of the IL-23/IL-22/AMP pathway during intestinal injury and advance the concept that IL-36 γ and IL-23 are fundamentally linked to repair of acute barrier damage. These findings provide new mechanistic insight into how the host commandeers pro-inflammatory cytokines for tissue repair and highlights the potential for manipulating the IL-36/IL-23/IL-22/AMP network in treating acute intestinal damage.

2.3 Introduction

At mucosal surfaces, particularly the intestine, epithelial cells form a physical and functional barrier that protects the host from the unrestricted barrage of microbial and environmental stimuli. Compromises in the epithelial barrier due to damage or dysfunction can result in activation of underlying immune cells. Once activated, innate and adaptive immune cells display enhanced anti-microbial activity and promote epithelial proliferation, repair of the damaged barrier, and resolution of inflammation [68]. However, if the insult persists, or if repair processes are ineffective, chronic intestinal inflammation as seen in human inflammatory bowel disease (IBD) may ensue [69]. Therefore, delineating the specific mechanisms involved in efficient tissue repair processes in the damaged intestine may provide insight into therapeutic strategies for the treatment of these inflammatory conditions.

Interleukin (IL)-22 is a key cytokine that links intestinal immune activation to epithelial repair and barrier protection following damage [68, 70]. IL-22 is expressed by numerous immune cells including type 3 innate lymphoid cells (ILC3), natural killer (NK) cells, neutrophils, and Th17 and Th22 cells [71]. Intestinal epithelial cells express the IL-22R complex and binding of IL-22 results in the induction of mucins, antimicrobial peptides, and anti-apoptotic pathways that collectively aid in limiting bacterial encroachment while promoting epithelial proliferation, wound healing and repair [72]. Mice that lack the ability to produce IL-22 following treatment with dextran sodium sulfate (DSS) or *Citrobacter rodentium* are grossly unable to repair barrier damage or control pathogenic bacterial expansion [73-75]. These data suggest that IL-22 plays a non-redundant function in mucosal barrier defense [76, 77].

Investigations into how IL-22 is regulated have led to the identification of IL-23 as one of the most potent inducers of this cytokine. Systemic administration of bacterial flagellin was shown to rapidly induce IL-23 production by intestinal TLR5 expressing CD103⁺CD11b⁺ dendritic cells (DCs) and subsequent IL-22 expression [78]. Additionally, stimulation of intestinal ILC3s, NK cells, neutrophils and Th17 cells with IL-23 potently induces IL-22 production [71]. Similarly, loss of IL-23 signaling *in vivo* during DSS-induced colitis completely abrogates colonic IL-22 expression and results in exacerbated disease [75]. Furthermore, IL-23p19-deficient mice fail to produce IL-22, which leads to overgrowth of segmented filamentous bacteria (SFB) [79]. Collectively, these studies demonstrate an important role for the IL-23/IL-22 axis in barrier protection and control of bacteria, yet the upstream regulators of this critical pathway are incompletely undefined.

Among the many immunological factors produced in response to intestinal damage, IL-1 superfamily cytokines appear to play a major role in the inflammatory program [80]. IL-1 β , IL-

18, and IL-33 are all induced during experimental colitis and are believed to contribute to the pathogenesis of IBD, but may also be involved in tissue protection [81-83]. Similarly, IL-36 cytokines, the more recently described members of IL-1 superfamily, appear to potently induce inflammatory responses and regulate mucosal immunity [49, 84]. We and others have reported that IL-36 cytokines are expressed in the intestine during inflammation [43, 55, 85-88] in response to stimulation by the microbiota [55]. Once expressed, IL-36 ligands are involved in the activation of innate and adaptive immune cells and stromal cells that can exacerbate intestinal inflammation, and also play an instrumental role in resolution of intestinal damage [55, 85, 89, 90]. This bi-functional effect of the IL-36/IL-36R axis during intestinal inflammation likely depends on the inducing stimuli, extent of tissue damage, and timing and chronicity of expression. In response to robust intestinal barrier destruction, IL-36R signals augment the inflammatory cascade early on, which appears linked to subsequent tissue protection and repair [55, 85]. However, the specific pathways via which IL-36R signaling controls host defense and barrier protection remain to be elucidated.

In this report, we define a critical IL-36/IL-23/IL-22 cytokine network that is instrumental for AMP production and host defense following intestinal damage. Using a murine model of colonic damage and inflammation, we show that IL-36 γ is a potent inducer of IL-23 production both *in vitro* and *in vivo*. IL-36 γ -induced IL-23 was highly dependent upon Notch2-dependent (CD11b⁺CD103⁺) DCs, but not CSFR1-dependent macrophages or Batf3-dependent (CD11b⁻CD103⁺) DCs. The intracellular signaling cascade linking IL-36R signaling to IL-23 production from DCs involved MyD88 and the NF-kB subunits c-Rel and p50. Consistent with *in vitro* observations, IL-36R-deficient mice exhibited dramatically reduced IL-23 and IL-22/AMP levels and consequently mice failed to recover from acute intestinal damage. Interestingly, impaired

recovery of mice deficient in IL-36R or IL-36 γ could be completely rescued by treatment with exogenous IL-23. This recovery was accompanied by a restoration of IL-22 and AMP expression in the colon. Collectively, these data define a cytokine network involving IL-36 γ , IL-23, and IL-22, that is activated in response to intestinal barrier damage and involved in providing critical host defense.

2.4 Material and methods

2.4.1 Mice

The following mice were obtained from Jackson Laboratories: Wild-type C57BL/6 (B6 WT), B6.129S(C)-*Batf3*^{tm1Kmm/J} (*batf3*^{-/-}), CD11c-cre (B6.Cg-Tg(Itgax-cre)1-1Reiz/J), *Notch2*^{fl/fl} (B6.129S-*Notch2*^{tm3Grid/J}), B6.129P-*Nfkb1*^{tm1Bal/J} (*p50*^{-/-}), and B6.129P2(SJL)-*Myd88*^{tm1.1Defr/J} (*myd88*^{-/-}). IL-36R^{-/-} mice (*Il1rl2*^{-/-}) on the C57BL/6 background (backcrossed >9 generations) were originally provided by Amgen. To generate IL-36 γ ^{-/-} (*Il1f9*^{-/-}) mice, sperm from IL-36 γ ^{-/-} mice was obtained from the KOMP repository (UC Davis) and heterozygous *Il1f9*^{+/-} founder mice were generated by the Mouse Transgenic and Gene Targeting Core facility at Emory University. *Il1f9*^{+/-} mice were subsequently bred to generate *Il1f9*^{-/-} mice on the C57BL/6 background (backcrossed >9 generations). *Notch2*^{CKO} mice were generated as previously described [91].

2.4.2 DSS model of colitis.

Mice were treated with 2.5% - 3% (wt/vol) DSS (MP Biomedicals; molecular weight: 36,000-50,000) in their drinking water for 5 days and then switched to regular drinking water. Mice were monitored daily for signs of diseases and DAI and histology scoring was performed as previously described [55].

2.4.3 Colonic explants

Colon tissue was harvested from mice, open longitudinally, and washed in phosphate buffered saline + Tween-20 (PBS-T). Biopsy punches (3mm; Integra Miltex, New York, NY, United States) were used to excise sections of the colon, which were placed in 96-well plates and cultured with RPMI-1640 supplemented with 10% fetal bovine serum and penicillin/streptomycin. Recombinant IL-23 (R&D system, Minneapolis, MN, United States) or recombinant IL-36 γ ((R&D system, Minneapolis, MN, United States) were added to each well at 20ng/mL and 100ng/mL, respectively. For gene expression analysis, tissues were collected and processed for downstream applications 6 hours following stimulation. For protein analysis, supernatant from the tissues were collected 60 hours after stimulation.

2.4.4 ELISA

IL-22 and IL-23 secretion was measured in cell-free culture supernatants using IL-22 and IL-23 ELISA kits (R&D system, Minneapolis, MN, United States) according to the manufacturer's protocols.

2.4.5 *In vivo* administration of IL-23

Recombinant mouse IL-23 was purchased from R&D Systems (Minneapolis, MN). *Il1rl2*^{-/-} and *Il1f9*^{-/-} mice received either PBS or 0.25ug of IL-23 via i.p. injection at day 3, 4 and 5 of DSS treatment.

2.4.6 Histology

Colons were fixed in 10% formalin. Paraffin embedding, sectioning, Hematoxylin/Eosin staining, and slide scanning was performed at the University of Michigan Pathology Core.

2.4.7 Bone marrow-derived dendritic cells.

Bone marrow-derived dendritic cells (BMDCs) were generated from 8-15 week-old B6 WT, *c-Rel*^{-/-} and *p50*^{-/-} mice. BM cells were harvested from femurs, and the red blood cells were lysed by using red blood cell lysis buffer. BM cells were cultured for 10 days in RPMI-1640 supplemented with 10% fetal bovine serum (FBS), penicillin/streptomycin, and 50μM β-mercaptoethanol. 20ng/mL of recombinant murine granulocyte macrophage colony-stimulating factor (Peprotech, Rocky Hill, NJ, United States) was added to cell cultures every two days. After 10 days of culture, the generated DCs were harvested. BMDCs (3x10⁵ cell/well) were seeded in 96-well plates and cultured ± IL-36γ (100ng/mL) for 6 hours or 24 hours. For inhibition of NF-κB components, BMDCs was pretreated with the c-Rel inhibitor IT-603 (Calibiochem, San Diego, CA, United States) or p50 inhibitor peptide or p65 inhibitor peptide (Novus biological, Littleton, CO, United States) for 1 hour ± IL-36γ. After 1 hour of pre-treatment, media containing inhibitors and IL-36γ was replaced with new media and incubated for 6 hours to analyze gene expression or 24 hours to analyze protein expression.

2.4.8 Quantitative real-time PCR

Total RNA was isolated from colon tissues of mice using the Qiagen RNeasy Mini Kit and QIAcube with on-column DNase digestion according to the manufacturer's protocol. cDNA was generated using the Superscript First Strand Synthesis kit for RT-PCR and random hexamer primers (Invitrogen). Q-PCR was performed with SYBR Green using a StepOnePlus PCR system (Applied Biosystem) and gene expression was normalized to *gapdh*.

2.4.9 Chromatin Immunoprecipitation (ChIP) assay

BMDCs were cultured ± IL-36γ (100ng/mL) for 8 hours. ChIP assays were performed using EZ-ChIP kit (EMD Millipore 17371) according to manufacturer's protocol. Briefly,

chromatin was obtained from the cultured cells after fixation with 18.5% formaldehyde and fragmented by sonication. One tenth of each fragmented chromatin sample was saved before immunoprecipitation for use as an input control. The remaining fragmented chromatin was immunoprecipitated with either control or anti-p50 (clone SC-8418) or anti-c-rel (clone sc-6955) antibodies (Santa Cruz Biotechnology). DNA recovered from the immunoprecipitation and input control were analyzed by qPCR.

2.4.10 Quantitation of fecal bacteria abundance.

Fecal samples were collected before DSS treatment and 6 days after beginning DSS treatment. All feces were stored at -80°C until DNA extraction. The total genomic DNA from each fecal sample was extracted using the QIAamp DNA stool minikit according to the manufacturer's protocol. Samples were analyzed for bacterial gene expression using the qPCR.

2.4.11 qPCR primer

All primers using for qPCR are listed below:

Il22

Fwd 5'-CAGGAGGTGGTGCCTTTCCT-3'

Rev 5'-TGGTCGTCACCGCTGATGT-3'

Il23

Fwd 5'-GCTGTGCCTAGGAGTAGCAG-3'

Rev 5'-TGGCTGTTGTCCTTGAGTCC-3'

Il1f6

Fwd 5'-TAGTGGGTGTAGTTCTGTAGTGTGC-3'

Rev 5'-GTTCTGTCTCAAGAGTGTCCAGATAT-3'

Il1f8

Fwd 5'-ACAAAAAGCCTTTCTGTTCTATCAT-3'

Rev 5'-CCATGTTGGATTTACTTCTCAGACT-3'

Il1f9

Fwd 5'-AGAGTAACCCCAGTCAGCGTG-3'

Rev 5'-AGGGTGGTGGTACAAATCCAA-3'

Il1rl2

Fwd 5'-AAACACCTAGCAAAAGCCCAG-3'

Rev 5'-AGACTGCCCGATTTTCCTATG-3'

Reg3a

Fwd 5'-GGCACCGAGCCCAATG-3'

Rev 5'-GGATTTCTCTCCCATGCAAAGT-3'

Reg3b

Fwd 5'-ATGCTGCTCTCCTGCCTGATG-3'

Rev 5'-CTAATGCGTGCGGAGGGTATATTC-3'

Reg3g

Fwd 5'-TTCCTGTCCTCCATGATCAAAA-3'

Rev 5'-CATCCACCTCTGTTGGGTTCA-3'

S100a8

Fwd 5'-TGAGTGTCTCAGTTTGTGCAG-3'

Rev 5'-TGTGAGATGCCACACCCACTTT-3'

S100a9

Fwd 5'-CAAATGGTGGAAGCACAGTTGGCA-3'

Rev 5'-TTGTGTCCAGGTCCTCCATGATGT-3'

Gapdh

Fwd 5'-CAAATGGTGGGAAGCACAGTTGGCA-3'

Rev 5'-TTGTGTCCAGGTCCTCCATGATGT-3

Occludin

Fwd 5'-TTGAAAGTCCACCTCCTTACAGA-3'

Rev 5'-CCGGATAAAAAGAGTACGCTGG-3'

Cldn2

Fwd 5'-GGCTGTTAGGCACATCCAT-3'

Rev 5'-TGGCACCAACATAGGAACTC-3'

Clostridium XIVa

Fwd 5'-AAATGACGGTACCTGACTAA-3'

Rev 5'-AAATGACGGTACCTGACTAA-3'

Clostridium XI

Fwd 5'-ACGGTACTTGAGGAGGA -3'

Rev 5'-GAGCCGTAGCCTTTCACT -3'

Helicobacter

Fwd 5'-ACCAAGGC(A/ T)ATGACGGGTATC-3'

Rev 5'-CGGAGTTAGCCGGTGCTTATT-3'

Desulfovibrio

Fwd 5'-CCGTAGATATCTGGAGGAACATCAG-3'

Rev 5'-ACATCTAGCATCCATCGTTTACAGC-3'

Oscillibacter

Fwd 5'-ACGGTACCCCTTGAATAAGCC-3'

Rev 5'-TCCCCGCACACCTAGTATTG-3'

Bacteroides

Fwd 5'-GAGAGGAAGGTCCCCCAC-3'

Rev 5'-CGCTACTTGGCTGGTTCAG-3'

Prevotella

Fwd 5'-CACCAAGGCGACGATCA-3'

Rev 5'-GGATAACGCCTGGACCT-3'

Alistipes

Fwd 5'-TTAGAGATGGGCATGCGTTGT-3'

Rev 5'-TGAATCCTCCGTATT-3'

Lactobacillus

Fwd 5'-AGCAGTAGGGAATCTTCCA-3'

Rev 5'-CACCGCTACACATGGAG-3'

2.4.12 Statistical analysis

All statistical analyses were performed with GraphPad Prism software, version 7.0 (Graphpad Software). ONE-way ANOVA and Tukey's Multiple Comparison Test or Student's *t* test were used to determine significance. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$; n.s.= not significant.

2.5 Results

2.5.1 *IL-36R-deficiency results in impaired IL-23 and IL-22 expression in the colons of DSS-treated mice.*

Recently, IL-36R signaling has been implicated in healing of mucosal damage [55, 85, 92] and our group demonstrated that IL-36R-deficient (*Il1rl2^{-/-}*) mice have impaired IL-22 production and consequently fail to recover from acute intestinal damage. In order to begin exploring potential mechanisms of how IL-36R signaling induces IL-22 expression, we performed a PCR array on total colonic tissues isolated from *Il1rl2^{+/+}* and *Il1rl2^{-/-}* mice at day 5 of DSS treatment. The array analysis revealed that the expression of IL-23 and IL-22 mRNA was ~9-fold and ~7-fold higher, respectively, in *Il1rl2^{+/+}* mice when compared to *Il1rl2^{-/-}* mice (Figure 2.1A). Given that IL-23 is a potent inducer of IL-22 [71], we postulated that impaired IL-22 expression in *Il1rl2^{-/-}* mice may be associated with a lack of IL-23. To explore the link between IL-36R signaling, IL-23, and IL-22, we first examined the expression of IL-23 and IL-22 in *Il1rl2^{+/+}* and *Il1rl2^{-/-}* mice during the course of DSS treatment (5 days DSS followed by 2 days of regular water). Quantitative PCR and ELISA analysis of colonic tissue revealed that DSS-induced expression of IL-23 and IL-22 mRNA and protein was significantly higher in colonic tissue isolated from *Il1rl2^{+/+}* mice when compared to *Il1rl2^{-/-}* mice (Figure 2.1 B-E). Additionally, following DSS treatment, the peak of IL-36 γ expression preceded that of IL-23 and IL-22 at day 3, followed by robust IL-23 expression on day 5 and IL-22 expression on day 7 (Figure 2.7 A). Collectively, these data suggest that signaling via IL-36R is involved in optimal IL-23 and IL-22 expression during DSS-induced damage.

2.5.2 *IL-36 γ -induced IL-22 production in colonic explants from DSS-treated mice is IL-23 dependent.*

Next, we determined whether IL-23 is required for IL-36 γ -induced IL-22 expression in colonic explants from DSS-treated mice. We focused our studies on IL-36 γ as it is the predominant IL-36 ligand produced in the colon of mice during DSS-induced damage [55]. Colonic explants isolated from healthy (non-DSS treated), wild type (WT) mice and stimulated with IL-36 γ *in vitro* showed no detectable increases in either IL-23 or IL-22. However, colonic explants isolated from DSS-treated mice on day 3, a time when endogenous IL-36 γ mRNA expression is highest (Figure 2.7 A and [55]), responded to exogenous IL-36 γ stimulation by inducing IL-23 (~3-fold) and IL-22 (~5-fold) compared to unstimulated controls (Figure 2.2 A and B). Of note, IL-36 α and IL-36 β were also capable of inducing IL-23 and IL-22 (Figure 2.7 B and C). These data further correlated with a strong induction of IL-36R (*Il1rl2*) mRNA expression at day 3 following DSS treatment (Figure 2.7 D). Having established that IL-36 γ is an inducer of IL-23, we next assessed whether IL-36 γ -induced IL-22 was IL-23-dependent. Indeed, antibody-mediated blockade of IL-23p19 (α p19) or IL-12/23p40 (α p40) was able to significantly reduce the ability of IL-36 γ to induce IL-22 in colonic explants from DSS-treated mice (**Figure 2.2 C**). Similarly, while colonic explants from DSS-treated IL-12/23p40-sufficient mice (*Il12b*^{+/+}) produced high levels of IL-22 in response to IL-36 γ stimulation, explants obtained from DSS-treated IL-12/23p40-deficient mice (*Il12b*^{-/-}) produced significantly less IL-22 under these conditions. This defect in IL-36 γ -induced IL-22 production in *Il12b*^{-/-} explant cultures was reversible by the addition of exogenous IL-23 (Figure 2.2 D). These results highlight a functional IL-36 γ /IL-23/IL-22 cytokine network in colonic tissue from DSS-treated mice.

2.5.3 *Notch2-dependent DCs are required for IL-36 γ -induced IL-23 and IL-22 expression and recovery from acute colonic damage.*

Having established IL-23 as a key intermediary between IL-36 γ and IL-22, we next examined whether specific antigen-presenting cell subsets may be involved in IL-23 induction in response to IL-36 γ . Intestinal DCs are categorized into two main populations: Notch2-dependent DCs and Batf3-dependent DCs [91]. Within these subsets, CD103⁺CD11b⁺ DCs have been reported to accumulate in the intestines during DSS-induced colitis [93]. To determine if either of these DC subsets are involved in IL-36 γ -induced IL-23 production, we used Notch2-floxed mice that had been crossed with CD11c-Cre mice to generate mice with a deletion of Notch2 in the DC lineage (*Notch2^{cKO}*), as well as Batf3-deficient mice (*batf3^{-/-}*). Initially, we examined the expression of IL-36R (*Il1rl2*) mRNA in colonic tissue isolated from these mice following treatment with DSS for 3 days. Consistent with Figure 2.7B, we found that *Il1rl2* was strongly induced in the colons of DSS-treated WT (*batf3^{+/+}*) mice, as well as in *batf3^{-/-}* mice. Conversely, the induction of *Il1rl2* was significantly reduced in the colons of DSS-treated *Notch2^{cKO}* mice, when compared to control mice (*Notch^{fl/fl}*) (Figure 2.3 A). Next, colonic explants from DSS-treated *batf3^{-/-}* and *Notch2^{cKO}* mice, and their respective controls, were stimulated *in vitro* with IL-36 γ and IL-23 as well as IL-22 expression was assessed by ELISA. While *batf3^{-/-}* mice exhibited normal induction of IL-23 and IL-22 in response to IL-36 γ , *Notch2^{cKO}* mice completely failed to produce IL-23 and IL-22 in the presence of IL-36 γ , when compared to when compared *Notch^{fl/fl}* controls. Furthermore, the addition of exogenous IL-23 to *Notch2^{cKO}* explant cultures was sufficient to restore IL-22 production in these cultures to normal levels (Figure 2.3 B and C). Thus, IL-36 γ -induced IL-23 appears to be dependent upon Notch2-dependent DCs *in vitro*. Of note, macrophages did not appear to play a significant role in IL-36 γ -induced IL-23 expression during

DSS since treatment of mice with α CSF-1R antibody to deplete macrophages [94] had no detectable effect on the ability of IL-36 γ to induce IL-23 or IL-22 in colonic explant cultures (Figure 2.8 A-C).

To investigate whether Notch2-dependent DCs are also necessary for host recovery from DSS-induced intestinal damage *in vivo*, the disease activity index (DAI) of *batf3*^{+/+}, *batf3*^{-/-}, *Notch2*^{*fl/fl*}, and *Notch2*^{*cKO*} mice was compared following administration of DSS in the drinking water for 5 days followed by normal water thereafter. While *batf3*^{+/+}, *batf3*^{-/-}, and *Notch2*^{*fl/fl*} mice were all able to recover normally from DSS-induced intestinal damage, *Notch2*^{*cKO*} mice were defective in colonic repair and had higher DAI scores (Figure 2.3 D), shorter colon length (Figure 2.3 E), and significantly reduced levels of IL-23 and IL-22 in colons directly *ex vivo* (Figure 2.3 F and G). Together, these data highlight Notch2-dependent DCs as a critical cellular source of IL-23 in response to IL-36 γ stimulation.

2.5.4 IL-36 γ induces IL-23 via signaling through MyD88, c-Rel and NF- κ Bp50.

MyD88 is an adaptor protein known to induce signaling through Toll-like receptors (TLRs) as well as IL-1 family receptors. To begin to define the signaling cascade linking IL-36R signaling to IL-23 expression in DCs, we generated bone marrow-derived DCs (BMDCs) from WT (*myd88*^{+/+}) and MyD88-deficient (*myd88*^{-/-}) mice and cultured them in the absence or presence of IL-36 γ . Upon stimulation of *myd88*^{+/+} BMDCs with IL-36 γ , IL-23 was robustly expressed, while *myd88*^{-/-} BMDCs completely failed to induce IL-23 protein secretion in response to IL-36 γ stimulation (Figure 2.9).

We next explored the involvement of the NF- κ B pathway in IL-36 γ -induced IL-23 expression. Since previous studies have implicated c-Rel in the expression of IL-23p19 to form functional IL-23 [95], we investigated the effects of c-Rel deficiency on IL-36 γ -induced IL-23

expression by using BMDC isolated from *c-rel*^{+/+} and *c-rel*^{-/-} mice. Following stimulation with IL-36 γ for 6 hours, we observed a strong induction of IL-23 from *c-rel*^{+/+} BMDCs, but no increase over baseline in *c-rel*^{-/-} cultures (Figure 2.4 A). Similarly, treatment with the c-Rel inhibitor IT-603 nearly completely abolished IL-36 γ -induced IL-23 expression (Figure 2.4 B).

Similar to the other components of the NF- κ B family of transcription factors, c-Rel complexes with proteins to facilitate downstream gene expression. Complexes of c-Rel can be either c-Rel/c-Rel, c-Rel/p50 or c-Rel/p65, so we sought to determine which NF- κ B proteins besides c-Rel may be involved in IL-36 γ -induced IL-23 expression. BMDCs from WT mice that were stimulated with IL-36 γ induced robust secretion of IL-23, and this effect was significantly inhibited (~60%) by p50 inhibitor peptide, but not p65 inhibitor peptide (Figure 2.4 C). Furthermore, BMDCs generated from NF- κ Bp50-deficient mice (*p50*^{-/-}) and stimulated with IL-36 γ showed significantly lower IL-23 expression (~3 fold) compared to those from WT (*p50*^{+/+}) mice (Figure 2.4 D). We next performed ChIP assays to assess p50 and c-Rel binding to the IL-23p19 promoter in BMDCs treated with IL-36 γ . As shown in Figure 2.4 E, there was a significant increase in c-Rel and p50 binding to the IL-23p19 promoter in response to treatment of BMDCs with IL-36 γ for 8 hours. Collectively, these results demonstrate that MyD88, c-Rel and NF- κ Bp50 are part of a signaling cascade downstream of IL-36R that is involved in IL-23 expression by DCs.

2.5.5 Systemic IL-23 administration promotes recovery from DSS-induced intestinal damage in IL-36R- and IL-36 γ -deficient mice in association with restoring IL-22 and anti-microbial peptide production.

Since IL-36 γ -induced IL-22 production in colonic explants from DSS-treated mice was IL-23 dependent (Figure 2.2 A), we next explored whether administration of IL-23 could rescue defective resolution of DSS-induced colonic damage in *Il1rl2*^{-/-} mice in association with restoring

IL-22 and AMP expression. DSS-treated *Il1rl2*^{-/-} mice received either PBS or IL-23 (0.25µg) at days 3, 4 and 5, and DSS was discontinued at day 5 and mice were switched to regular drinking water to monitor recovery from DSS-induced intestinal damage. Strikingly, systemic administration of IL-23 to DSS-treated *Il1rl2*^{-/-} mice was sufficient to promote full resolution of intestinal damage as DAI, colon length, and histology were similar to that observed in DSS-treated *Il1rl2*^{+/+} mice (Figure 2.5 A-E).

DSS induces massive damage to the intestinal epithelial barrier that allows microbes from the gut lumen to enter the underlying lamina propria. The physiological immune response to this damage is the induction of IL-22 and AMPs including S100A8, S100A9 and members of the Reg3 family (Reg3α, Reg3β, Reg3γ) [96]. Since IL-22 and AMPs are critically important in resolution of DSS-induced intestinal damage, we next examined if *Il1rl2*^{-/-} mice were defective in AMP expression following treatment with DSS and if this could be reversed by IL-23 administration. Following DSS-treatment for 5 days, *Il1rl2*^{+/+} mice expressed high levels of IL-22 and AMPs, particularly, S100A8, Reg3β, and Reg3γ. Consistent with their inability to induce IL-22 (Figure 2.6 A), *Il1rl2*^{-/-} mice were significantly impaired in S100A8, Reg3β, and Reg3γ mRNA expression in response to DSS-treatment, and this defect was nearly completely reversible to administration of IL-23. Indeed, delivery of IL-23 to *Il1rl2*^{-/-} mice was sufficient to induce S100A8, Reg3β, and Reg3γ to normal levels detected in *Il1rl2*^{+/+} mice (Figure 2.6 B-E).

Having observed that *Notch2*^{cKO} mice, like *Il1rl2*^{-/-} mice, failed to recover from DSS-induced intestinal damage and failed to express IL-23 and IL-22 *in vitro*, we next attempted to rescue these mice by injecting IL-23. The administration of IL-23 to *Notch2*^{cKO} mice was able to significantly reduce DAI and restore colon length (Figure 2.10 A and B) while also normalizing tissue architecture and histology scores to levels detected in *Notch2*^{fl/fl} mice (Figure 2.10 C and D).

IL-23 treatment of *Notch2^{CKO}* mice also induced IL-22 expression to similar levels as seen in control *Notch2^{fl/fl}* mice (Figure 2.10 E). Furthermore, delivery of IL-23 to *Notch2^{CKO}* mice was sufficient to induce Reg3 β and Reg3 γ (Figure 2.10 F and G).

We next sought to determine if our observations using *Il1rl2^{-/-}* mice were predominantly due loss of IL-36 γ signaling, or if other IL-36 agonist cytokines (IL-36 α , IL-36 β) could be playing a role. To do so, we first treated IL-36 γ -deficient mice (*Il1f9^{-/-}*) and control mice (*Il1f9^{+/+}*) with DSS and at day 3, colonic tissues were harvested and analyzed for IL-36 α , IL-36 β and IL-36 γ expression. In the absence of DSS, IL-36 α and IL-36 β were not detectable in *Il1f9^{+/+}* and *Il1f9^{-/-}* mice, while DSS-treatment of *Il1f9^{+/+}* mice led to the robust expression of IL-36 γ , and only very low levels of IL-36 α and undetectable levels of IL-36 β expression. As expected, *Il1f9^{-/-}* mice had undetectable levels of IL-36 γ and did not appear to induce IL-36 α or IL-36 β to compensate for the loss of IL-36 γ (Figure 2.11 A). Interestingly, *Il1f9^{-/-}* mice appeared to phenocopy *Il1rl2^{-/-}* mice in response to DSS treatment in that they exhibited grossly impaired IL-23 and IL-22 production in colonic explants when compared to control *Il1f9^{+/+}* mice, and this defect could be overcome by the addition of IL-36 γ (Figure 2.11 B and C). To extend these *in vitro* observations to the *in vivo* setting, *Il1f9^{-/-}* mice were treated with DSS in the presence or absence of IL-23 administration as in Figure 2.6. Similar to effects observed in *Il1rl2^{-/-}* mice, treatment of *Il1f9^{-/-}* mice with IL-23 was able to significantly reduce DAI (Figure 2.12 A) and normalize colon length (Figure 2.12 B and C) and histological damage to that seen in control *Il1f9^{+/+}* mice (Figure 2.12 D and E). IL-23 treatment of *Il1f9^{-/-}* mice further restored IL-22 (Figure 2.13 A) and AMP expression, particularly S100A8, Reg3 β , and Reg3 γ , back to levels observed in *Il1f9^{+/+}* mice (Figure 2.13 B-F). Collectively, these results demonstrate that delivery of IL-23 to *Il1rl2^{-/-}* mice and *Il1f9^{-/-}* mice is sufficient to restore IL-22 and AMP expression and recovery from acute intestinal damage.

Since intercellular tight junctions are essential for maintaining the integrity and function of the intestinal barrier in the steady-state and following damage [97], we next assessed whether the mRNA expression of the tight junction components occludin and claudin 2 were affected by the loss of IL-36 signaling. Following treatment with DSS for 5 days, both *Il1rl2*^{-/-} and *Il1f9*^{-/-} mice exhibited significantly reduced occludin and claudin 2 mRNA expression, when compared to wild-type controls. Additionally, IL-23 administration was able to restore occludin and claudin 2 mRNA expression to wild-type levels (Figure 2.14 A-D). These data suggest that signaling via IL-36R not only induces IL-23-dependent mucosal protection via IL-22 and AMP expression, but also may help to reseal the damaged intestinal epithelial barrier via effects on tight junctions.

Given the dynamic crosstalk between the mucosal immune system and the gut microbiota [98-100], combined with the established involvement of IL-23, IL-22, and AMPs in controlling the microbiota [101, 102], we next explored the contribution of the IL-36/IL-36R axis in regulating microbiota composition. In the steady state, we observed that *Il1rl2*^{-/-} mice had a significant increase in several flagellated bacterial groups including Clostridium cluster XIVa and XI and Oscillibacter, and significant decreases in the non-flagellated bacterial groups Bacteroides, Prevotella, and Lactobacillus (Figure 2.15 A). Interestingly, most of these changes were further augmented upon DSS treatment (Figure 2.15 B). These observations support a potential role for the IL-36/IL-23/IL-22/AMP axis in control of the microbiota during health and disease and future studies using 16S rRNA sequencing should further clarify the full extent to which this cytokine axis influences the microbiota.

2.6 Discussion

In this study, we provide evidence demonstrating that the IL-36/IL-36R pathway acts as a key upstream inducer of IL-23/IL-22/AMP-dependent colonic tissue repair. While IL-36 ligands

are well-appreciated to promote chronic inflammation and contribute to pathological tissue damage [49, 83, 103], their role in mediating tissue protection in response to acute insult is newly emerging [55, 85, 92]. We and others have recently reported that IL-36R-deficient mice treated with DSS have reduced signs of intestinal inflammation during the damage phase of disease, yet are impaired in mucosal healing [43, 55, 85]. These data suggest that the pro-inflammatory functions of the IL-36 pathway are intimately linked with epithelial regeneration, tissue repair, and healing of intestinal damage and act as part of a feedback loop that then limits further production of pro-inflammatory factors and pathological inflammation. IL-36 cytokines may likewise function to promote wound repair at other barrier surfaces, such as the skin. In this regard, a recent report observed that IL-36 γ was induced in a model of skin injury and signaling via IL-36R promoted wound healing via the induction of Reg3 γ [92]. Whether or not IL-23 and/or IL-22 were also involved in IL-36R-mediated wound repair in this skin model remains an open question.

The IL-36/IL-23/IL-22 inflammatory cytokine cascade in response to DSS-mediated intestinal injury is a highly orchestrated process that involves numerous innate immune cell subsets. Early following DSS-induced damage, inflammatory monocytes/macrophages are a main source of IL-36 γ in response to components of the microbiota [55], however keratinocytes [104], myofibroblasts [89, 90], and other cells types may also be important sources of IL-36 cytokines [87]. While many cell types express IL-36R, we found that IL-36R expression was dramatically increased early following DSS treatment at a time that coincided with accumulation of CD11b⁺CD103⁺ DCs. Furthermore, in *Notch2*^{CKO} mice that lack CD11b⁺CD103⁺ DCs, IL-36R expression was not increased following DSS treatment suggesting that Notch2-dependent CD103⁺CD11b⁺ DCs are recruited into the inflamed colon where they express high levels of IL-36R and produce IL-23 in response to IL-36 γ stimulation. These data are consistent with several

reports demonstrating that CD103⁺CD11b⁺ DCs are a main source of IL-23 [78, 105], and now directly link IL-36R signaling to IL-23 production by these cells for the first time. Following secretion of IL-23, numerous cell types in the colon express IL-23R [106-108] and are capable of producing IL-22 [68, 71, 109]. While ILC3s are the most well-documented IL-22 producers in the gut [70, 110, 111], activated neutrophils [55, 75, 112] and NK cells [73] can also produce IL-22 in response to IL-23 stimulation, and the relative contribution of IL-22 from these sources during intestinal damage and repair remain unclear. Additionally, IL-23R signaling directly in intestinal epithelial cells was recently shown to induce Reg3 β and CXCL1 expression, and the recruitment and activation of IL-22-producing neutrophils [113]. Regardless of the source, IL-22 is a potent inducer of epithelial proliferation, mucus production, and anti-microbial peptide expression, all of which support efficient intestinal tissue repair [76, 114, 115].

Several lines of evidence are consistent with the IL-36 γ /IL-36R axis playing a central role in IL-23/IL-22/AMP-dependent resolution of acute intestinal damage. First, IL-36R and IL-36 γ -deficient mice are grossly impaired in their ability to recover from DSS-induced intestinal damage and this phenotype can be rescued by treatment with IL-23 as well as an IL-22-inducing aryl-hydrocarbon receptor agonist [55]. Second, mice deficient in IL-23 [116] and IL-22 [73] appear to phenocopy the defective tissue repair in response to DSS that we observed in IL-36R and IL-36 γ -deficient mice. Third, *Notch2*^{cKO} mice, which lack CD103⁺CD11b⁺ DCs are also defective in colonic repair, an effect that can also be rescued by treatment with IL-23. Of note, the defective repair in *Notch2*^{cKO} mice was not as profound as that in IL-36R and IL-36 γ -deficient mice, suggesting that other cell types aside from CD103⁺CD11b⁺ DCs may also be involved in IL-36 γ -induced IL-23 production.

Both IL-36 cytokines and IL-23 are potent inflammatory cytokines that function in a context-dependent manner. In models of acute barrier damage that predominantly involve innate immune activation, the pro-resolution functions of these cytokines likely dominate over their pro-inflammatory effects on T cells and the net result may be beneficial to the host. Alternatively, in chronic conditions where T cells play a major role, pro-inflammatory effects of IL-36 cytokines and IL-23 may dominate over barrier protective effects and exacerbate disease pathology. This appears evident during skin and intestinal inflammation where CD4⁺ T cells are involved [54, 84, 87, 117-119]. Importantly, monoclonal antibody-mediated blockade of the p40 subunit of IL-12 and IL-23 is approved for the treatment of moderately to severely active Crohn's disease, and specific IL-23 blockers are showing efficacy in clinical trials [120]. Thus, our data demonstrating that the IL-36/IL-36R axis augments IL-23 expression in the intestine may inform on potential therapeutic targeting of IL-36 cytokines and/or IL-36R as a novel strategy to limit pro-inflammatory effects of IL-23 during human IBD.

The context-dependent role of IL-36 cytokines in inducing pro-inflammatory responses that lead to intestinal barrier protection appears to be an emerging paradigm for members of the IL-1 family cytokines [81, 82, 121]. While IL-1 α augments colonic inflammation, IL-1 β is involved in restitution of the epithelial barrier and resolution of acute colonic damage [122]. Similarly, NLRP6, ASC, caspase-1, and IL-18 are all protective in the DSS model of colitis [123, 124]. The alarmin IL-33 can also promotes intestinal tissue protection via the amphiregulin-EGFR pathway and act on ST2-expressing regulatory T cells to promote their function in suppression of colitis [125]. IL-37 is an atypical member of the IL-1 family in that it functions as an inhibitor of innate inflammation and immunity, yet still functions to protect from colitis in mice [126, 127]. Our data indicate that IL-36 γ and IL-36R are rapidly induced following acute colonic damage and

orchestrate a key inflammatory process involving CD103⁺CD11b⁺ DCs, IL-23, IL-22, and AMPs, which ultimately functions to resolve colonic damage and provide host protection (Figure 2.16). These findings have potential implications for the treatment of intestinal inflammatory conditions, including IBD, where the beneficial effects of IL-36 and/or IL-23 blockade may be limited by concomitant interference with tissue repair processes. Therefore, a combined therapeutic approach aimed at inhibiting pro-inflammatory cytokines, while augmenting tissue repair mechanisms may afford optimal treatment for chronic intestinal inflammation.

2.7 Figures and figure legends

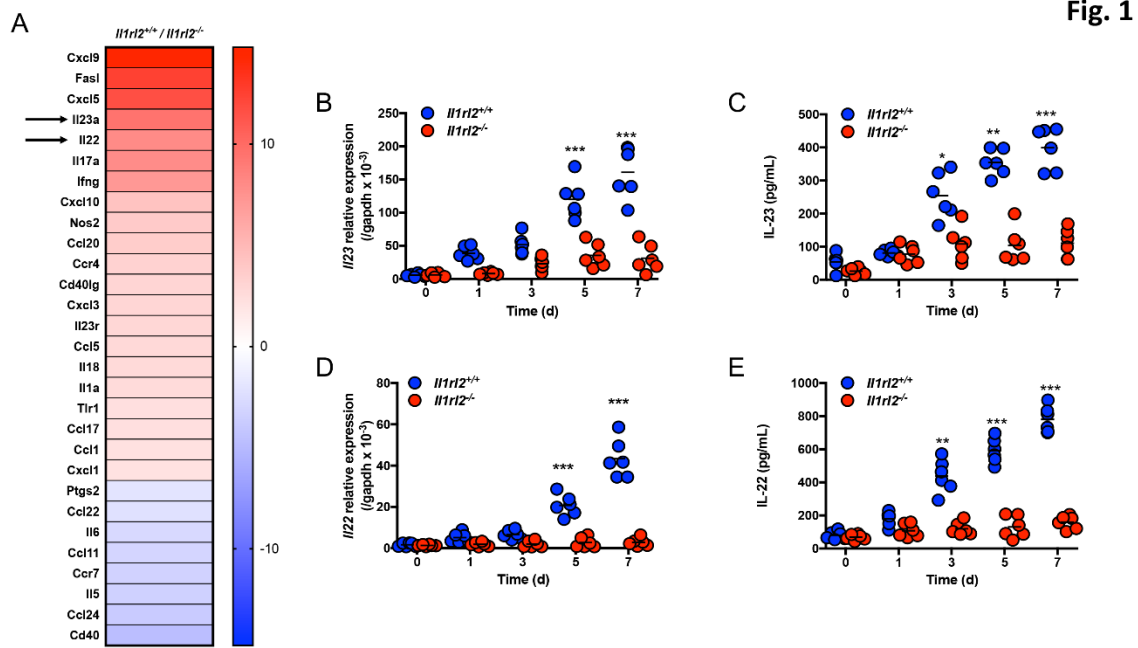


Figure 2.1 IL-36R-deficiency results in impaired IL-23 and IL-22 expression in the colons of DSS-treated mice.

(A) PCR array gene expression analyses from colon tissues of *Il1rl2*^{+/+} and *Il1rl2*^{-/-} mice treated with DSS for 5 days. (B-C) Time course of IL-23 mRNA (B) and protein (C) expression in colons from *Il1rl2*^{+/+} and *Il1rl2*^{-/-} mice treated with DSS. (D-E) Time course of IL-22 mRNA (D) and protein (E) expression in colons from *Il1rl2*^{+/+} and *Il1rl2*^{-/-} mice treated with DSS. Data are representative of 3 independent experiments with 3-4 mice/group. All data are presented as mean \pm SEM; * P < 0.5, ** P < 0.05, *** P < 0.001.

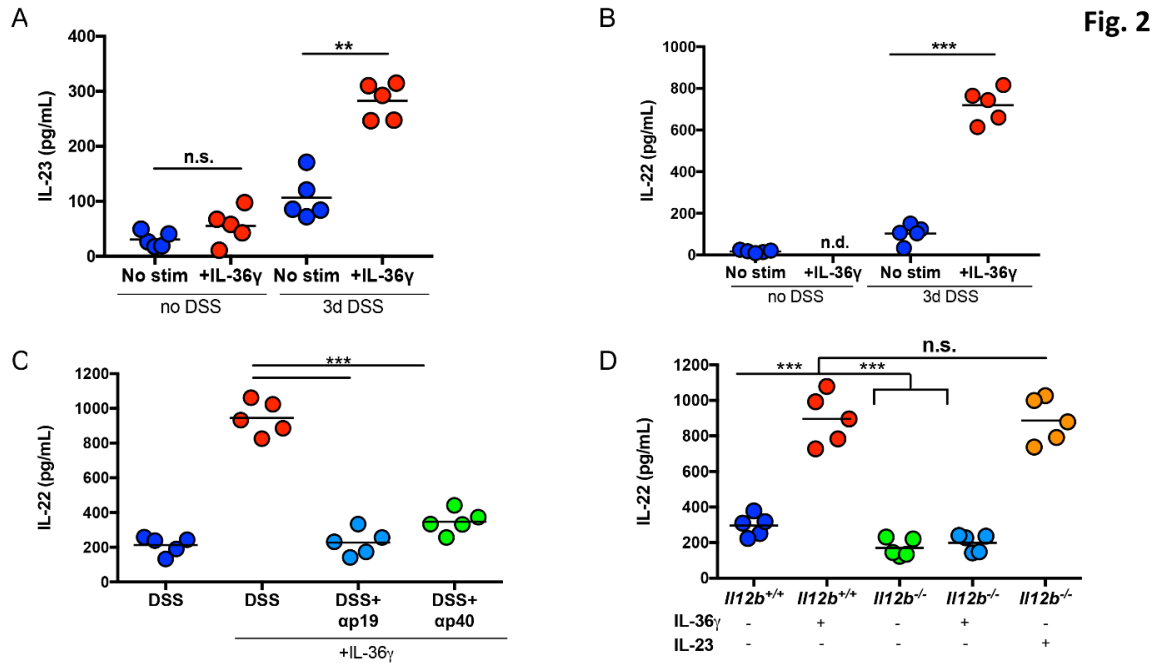


Figure 2.2 IL-36 γ -induced IL-22 production in colonic explants from DSS-treated mice is IL-23 dependent.

(A-B) Colonic explants from control (no DSS) or 3-day DSS-treated (3d DSS) WT mice were cultured for 60h in the presence or absence of IL-36 γ . (A) Supernatants were analyzed for IL-23 (A) and IL-22 (B) by ELISA. (C) Colonic explants from 3-day DSS-treated WT mice were stimulated with IL-36 γ and α p19 or α p40 antibodies for 60h and IL-22 expression was assessed by ELISA. (D) Colonic explants from 3-day DSS-treated WT (*Il12b*^{+/+}) or *Il12b*^{-/-} mice stimulated with IL-36 γ or IL-23 for 60h and IL-22 expression was assessed by ELISA. Data are representative of at 2 independent experiments with 4-5 mice/group. All data are presented as mean \pm SEM; n.s.= not significant; ** P < 0.01, and *** P < 0.001, one-way ANOVA with Tukey's multiple comparison test.

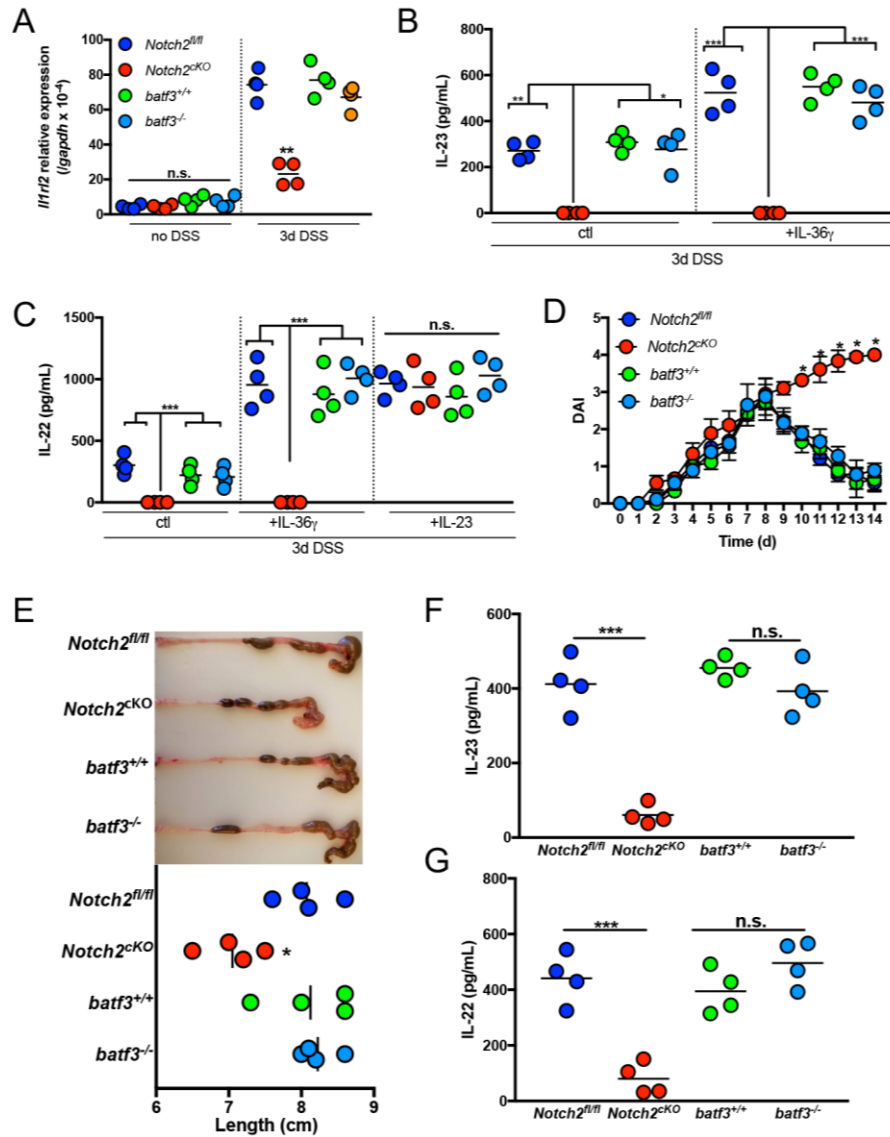


Figure 2.3 Notch2-dependent DCs are required for IL-36 γ -induced IL-23 and IL-22 expression and recovery from colonic damage.

(A) IL-36R (*Il1rl2*) mRNA expression was analyzed by qPCR in colon tissue isolated from DSS-treated *batf3^{+/+}*, *batf3^{-/-}*, *Notch2^{fl/fl}* and *Notch2^{CKO}* mice directly *ex vivo*. (B-C) Colonic explants from DSS-treated mice were cultured for 60h in the presence or absence of IL-36 γ or IL-23. Supernatants were analyzed for IL-23 (B) and IL-22 (C) expression by ELISA. (D) DAI of *batf3^{+/+}*, *batf3^{-/-}*, *Notch2^{fl/fl}* and *Notch2^{CKO}* mice treated with DSS for 5 days, followed by normal water. (E) Image and colon length from mice treated as in (D), at day 14. (F-G) Expression of IL-23 (F) and IL-22 (G) in colon tissues from DSS mice at day 5. Data are representative of 2 independent experiments with 3-4 mice/group. All data are presented as mean \pm SEM; n.s.= not significant; * $P < 0.05$, ** $P < 0.05$, *** $P < 0.001$, one-way ANOVA with Tukey's multiple comparison test.

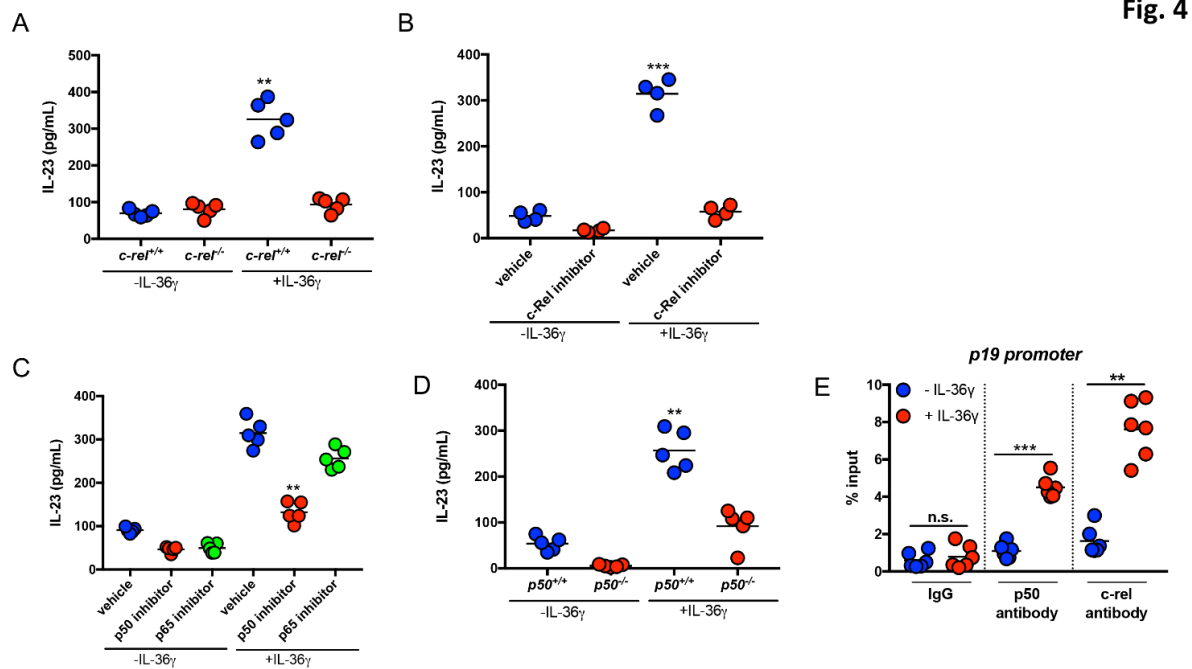


Figure 2.4 IL-36 γ induces IL-23 via signaling through c-Rel and NF- κ Bp50

(A) BMDC were generated from *c-rel*^{+/+} and *c-rel*^{-/-} mice and cultured in the presence or absence of IL-36 γ for 24 hr and IL-23 was assessed by ELISA. (B-C) WT BMDC were cultured in the presence or absence of IL-36 γ for

24 hr and IL-23 was assessed by ELISA. (B) Some cultures were pre-treated with the c-Rel inhibitor (IT-603) or with vehicle control (DMSO) for 1h. (C) Some cultures were pre-treated with p50 or p65 inhibitor peptides or control peptides for 1h. (D) BMDC were generated from *p50*^{+/+} and *p50*^{-/-} mice and cultured in the presence or absence of IL-36 γ for 24 hr and IL-23 was assessed by ELISA. (E) ChIP assays for p50 and c-Rel binding to the p19 promoter in BMDCs treated with IL-36 γ for 8 h. Data in (A-D) are representative of at least 2 independent experiments with $n = 5$. Data in (E) are the combined data of two independent experiments with 3 replicates per experiment. All data are presented as mean \pm SEM; ** $P < 0.01$, and *** $P < 0.001$, one-way ANOVA with Tukey's multiple comparison test.

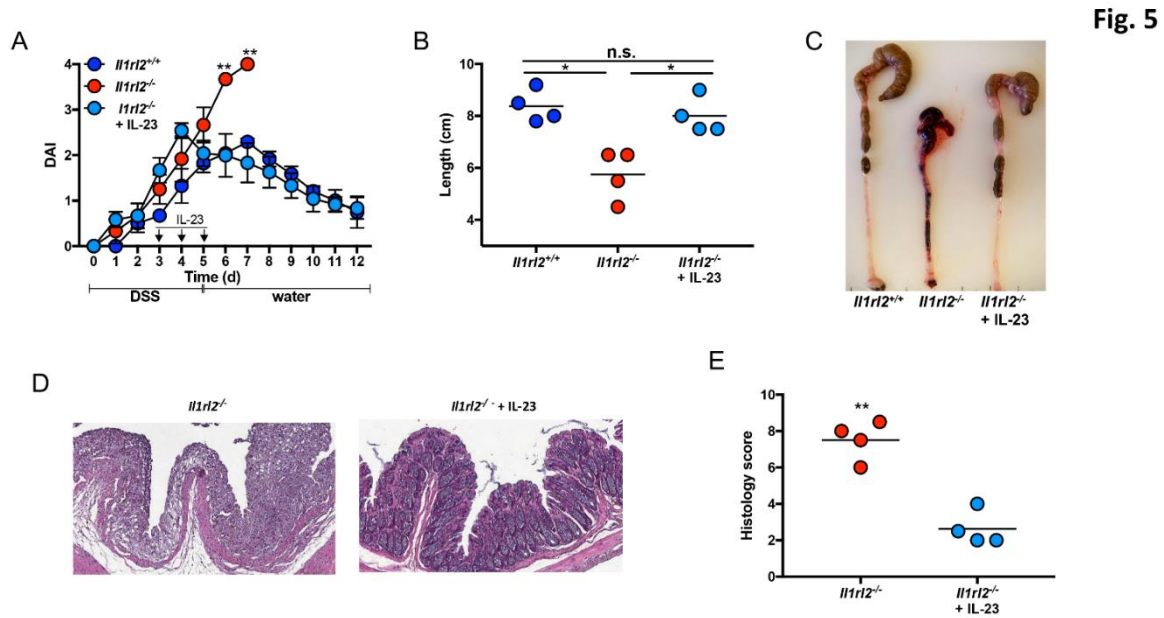


Figure 2.5 Systemic IL-23 administration induces resolution of DSS-induced colonic damage in *Il1rl2*^{-/-} mice.

(A) DAI of *Il1rl2*^{+/+} and *Il1rl2*^{-/-} mice treated with DSS for 5 days, followed by normal water for 7d, in the presence or absence of IL-23. (B-C) Image and length of colons from mice treated as in (A). (D) H&E staining and (E) histology scoring of colon sections from mice treated as in (A). Data are representative of 3 independent experiments with 4-5 mice/group. All data are presented as mean \pm SEM; n.s.= not significant; * $P < 0.05$, ** $P < 0.01$, one-way ANOVA with Tukey's multiple comparison test.

Fig. 6

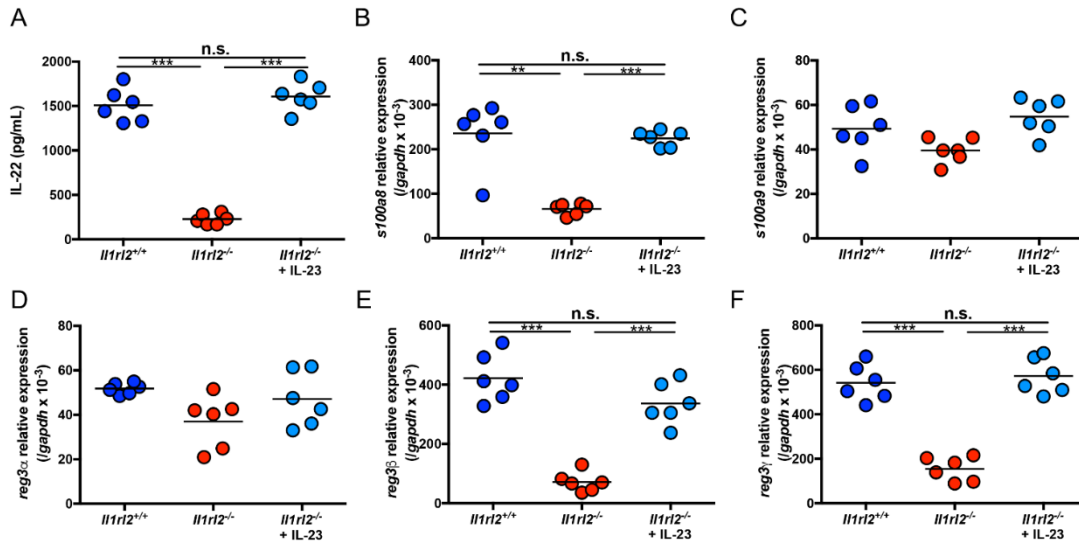


Figure 2.6 Systemic IL-23 administration induces IL-22 and AMPs and rescues *Il1rl2*^{-/-} mice from DSS-induced colonic damage.

(A) IL-22 protein expression in colons from *Il1rl2*^{+/+} and *Il1rl2*^{-/-} mice treated with DSS for 5 days in the presence or absence of IL-23. (B) S100A8 (C) S100A9 (D) Reg3α (E) Reg3β and (F) Reg3γ mRNA expression in colons isolated from mice as in (A). Data are representative of 2 independent experiments with 5-6 mice/group. All data are presented as mean ± SEM; n.s.= not significant; ***P* < 0.01, and ****P* < 0.001, one-way ANOVA with Tukey's multiple comparison test.

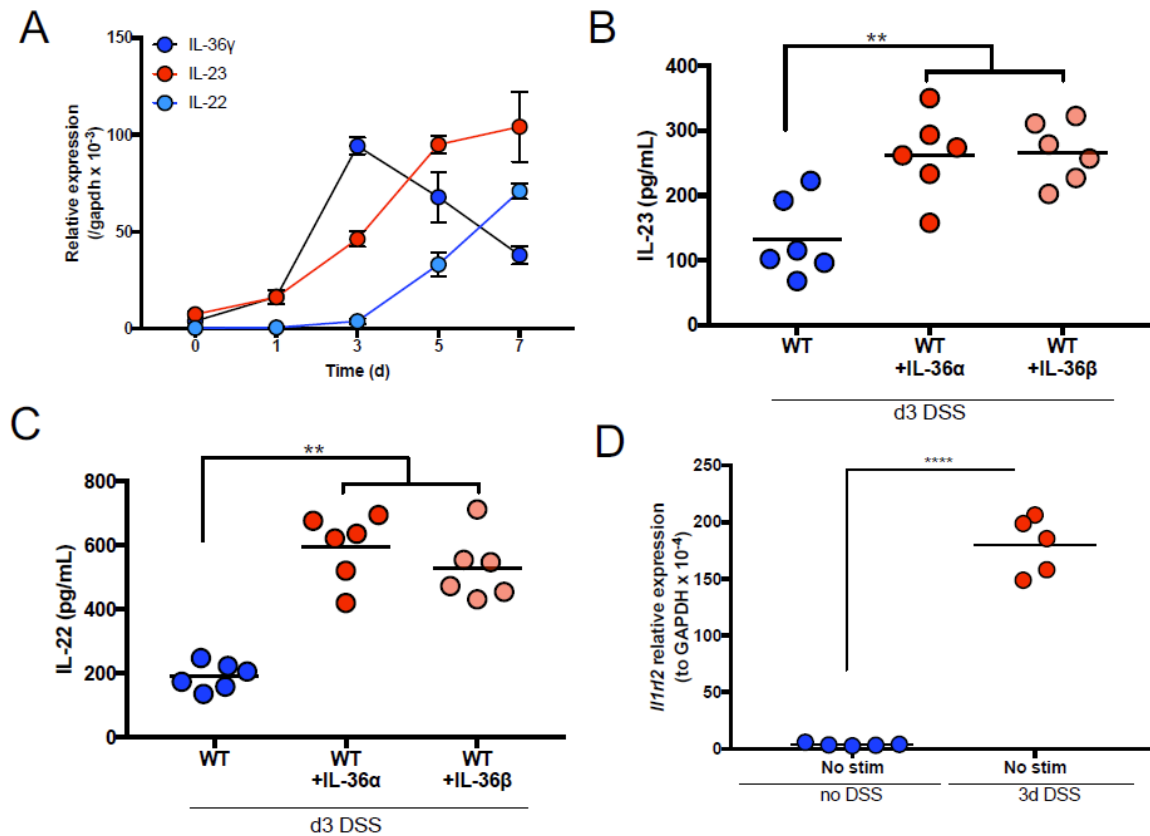


Figure 2.7 Chapter 2 S1. Expression of IL-36 γ , IL-23, IL-22 and IL-36R during DSS treatment.

(A) Time course analysis of IL-36 γ , IL-23, and IL-22 mRNA expression in colonic tissue isolated from WT mice during the course of DSS treatment (5 days DSS followed by 2 days of regular water). (B-C) Colonic explants from DSS-treated mice at day 3 DSS stimulated with IL-36 α and IL-36 β for 60h and supernatants were analyzed for IL-23. (B) and IL-22 (C) by ELISA. (D) qPCR analysis of *Il1r2* expression in colonic explants isolated from DSS-treated mice as in (B-C). Data are representative of at least 2 independent experiments with 4-5 mice/group. All data are presented as mean \pm SEM; ** $P < 0.01$ and **** $P < 0.0001$, Student's unpaired t test.

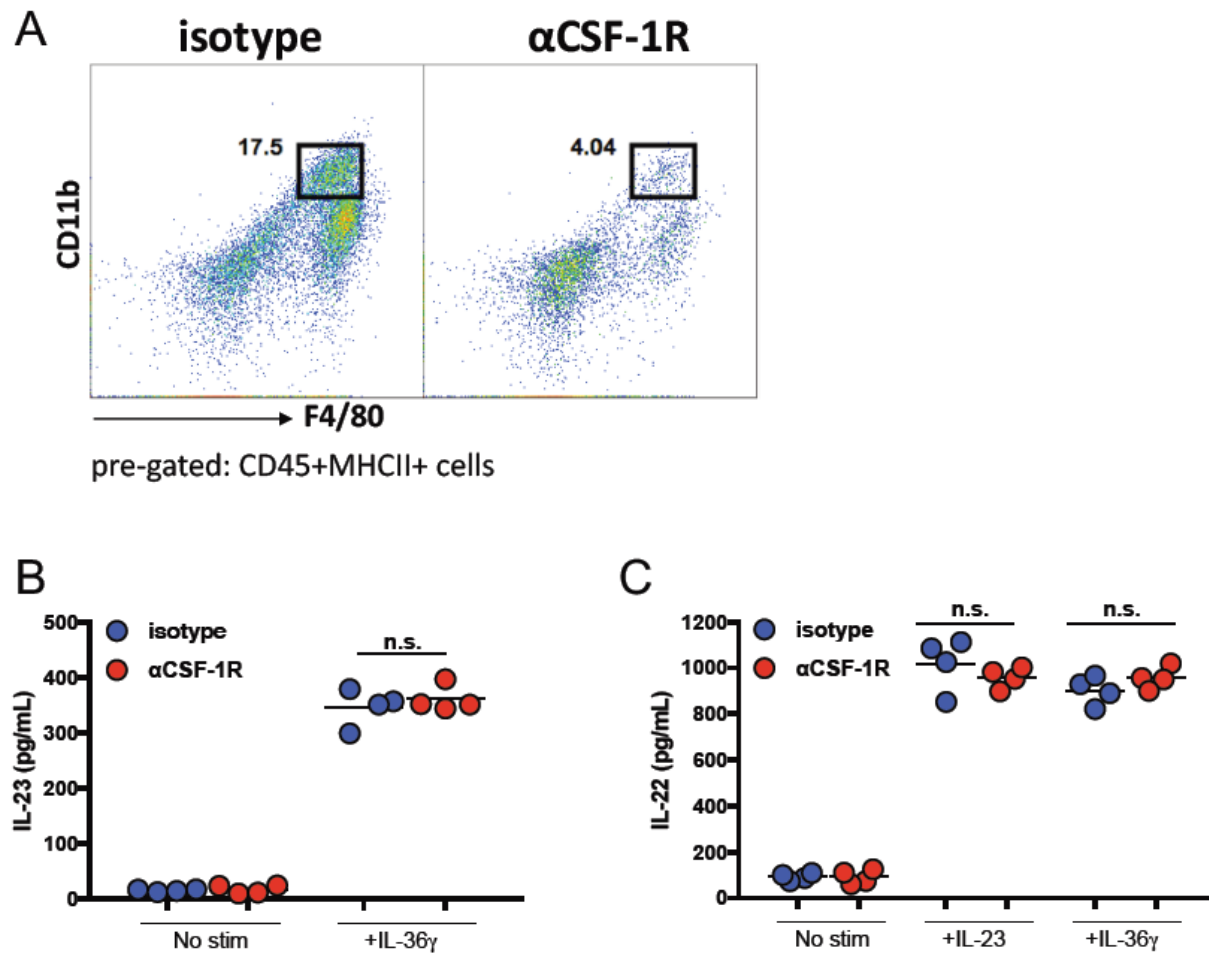


Figure 2.8 Injection of anti-CSF-1R antibody does not alter IL-36 γ -induced IL-23 or IL-22 production.

(A) FACS plots of total colonic lamina propria cells isolated from WT mice treated with neutralizing anti-CSF-1R antibody or isotype control antibody and analyzed at day 3 of DSS treatment. (B-C) Colonic explants from DSS-treated mice as in (A) were cultured for 60h in the presence or absence of IL-36 γ or IL-23. Supernatants were analyzed for IL-23 (B) and IL-22 (C) expression by ELISA. All data are presented as mean \pm SEM; n.s.= not significant; one-way ANOVA with Tukey's multiple comparison test.

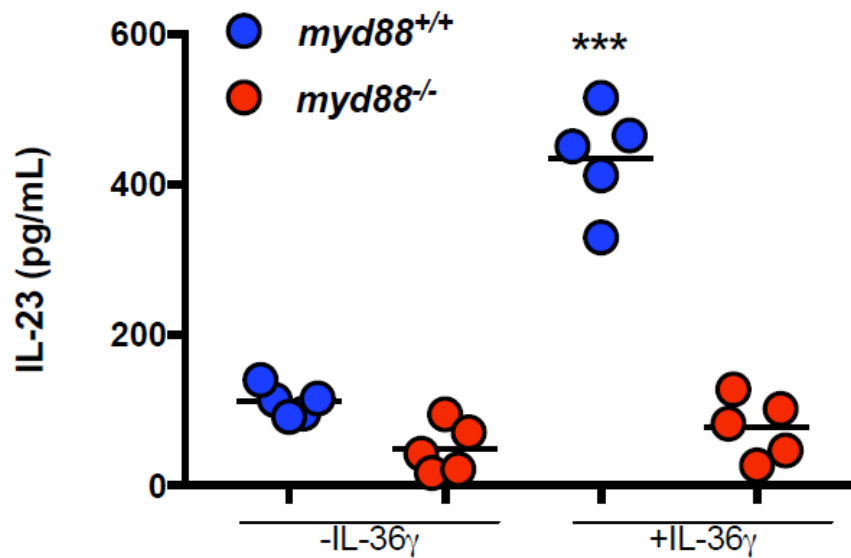


Figure 2.9 IL-36 γ signaling stimulates IL-23 production in BMDCs via MyD88.

WT (*myd88*^{+/+}) or *myd88*^{-/-} BMDCs were cultured for 60h in the presence or absence of IL-36 γ . Supernatants were analyzed for IL-23 by ELISA. Data are representative of 2 independent experiments with 4-5 mice/group. All data are presented as mean \pm SEM; ****P* < 0.001, one-way ANOVA with Tukey's multiple comparison test.

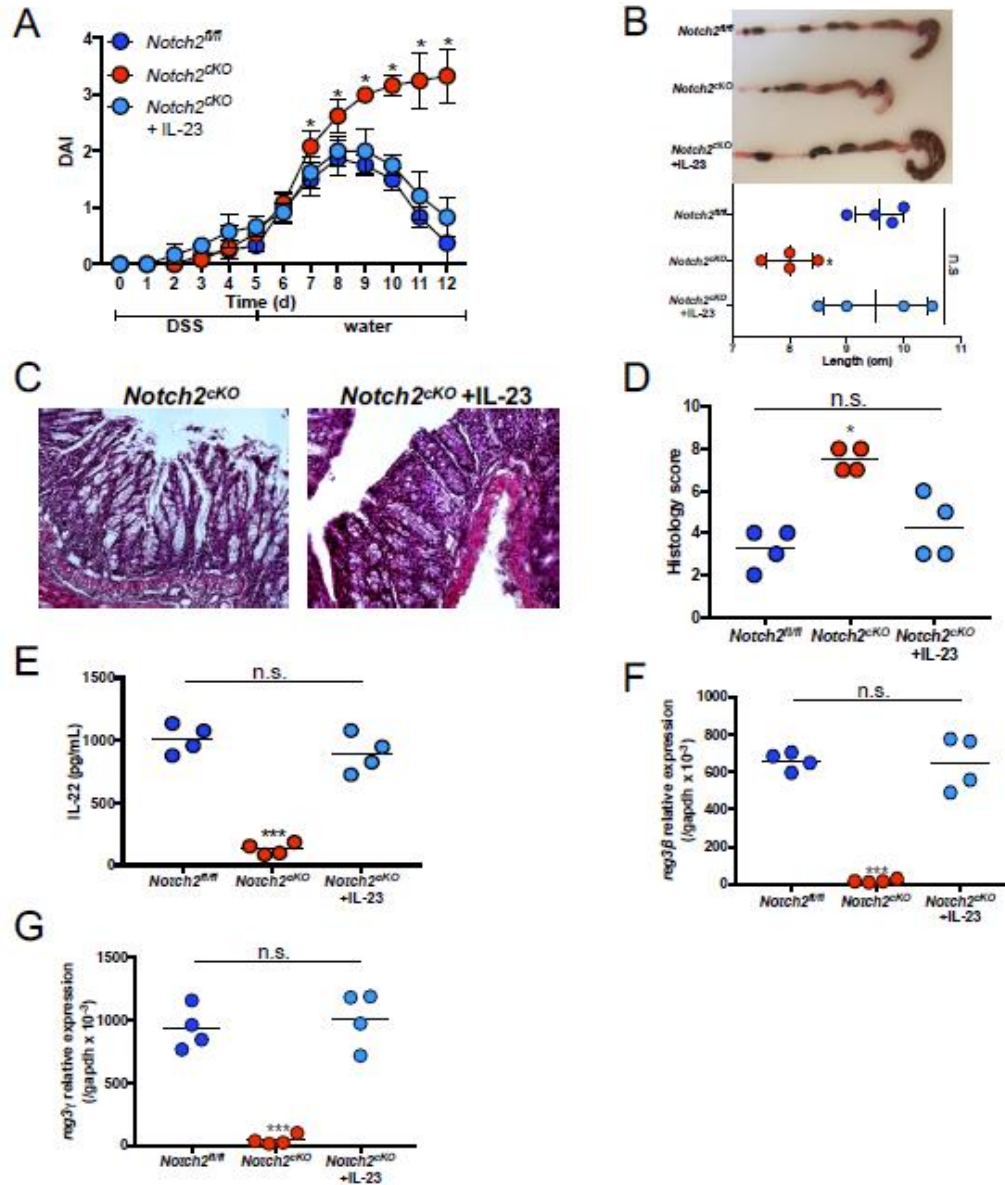


Figure 2.10 Systemic IL-23 administration induces the resolution of DSS-induced colonic damage in *Notch2cKO* mice.

(A) DAI of *Notch^{fl/fl}* and *Notch2^{cKO}* mice treated with DSS for 5 days, followed by normal water for 7 days, in the presence or absence of IL-23. (B) Image and length of colons from mice treated as in (A). (C) H&E staining and (D) histology scoring of colon sections from mice treated as in (A). (E) IL-22 protein expression in colons from *Notch^{fl/fl}* and *Notch2^{cKO}* mice treated with DSS for 5 days in the presence or absence of IL-23. (F) Reg3 β and (G) Reg3 γ mRNA expression in colons isolated from mice as in (A). Data are representative of 3 independent experiments with 4-5 mice/group. All data are presented as mean \pm SEM; n.s.= not significant; * $P < 0.05$ and *** $P < 0.001$, one-way ANOVA with Tukey's multiple comparison test.

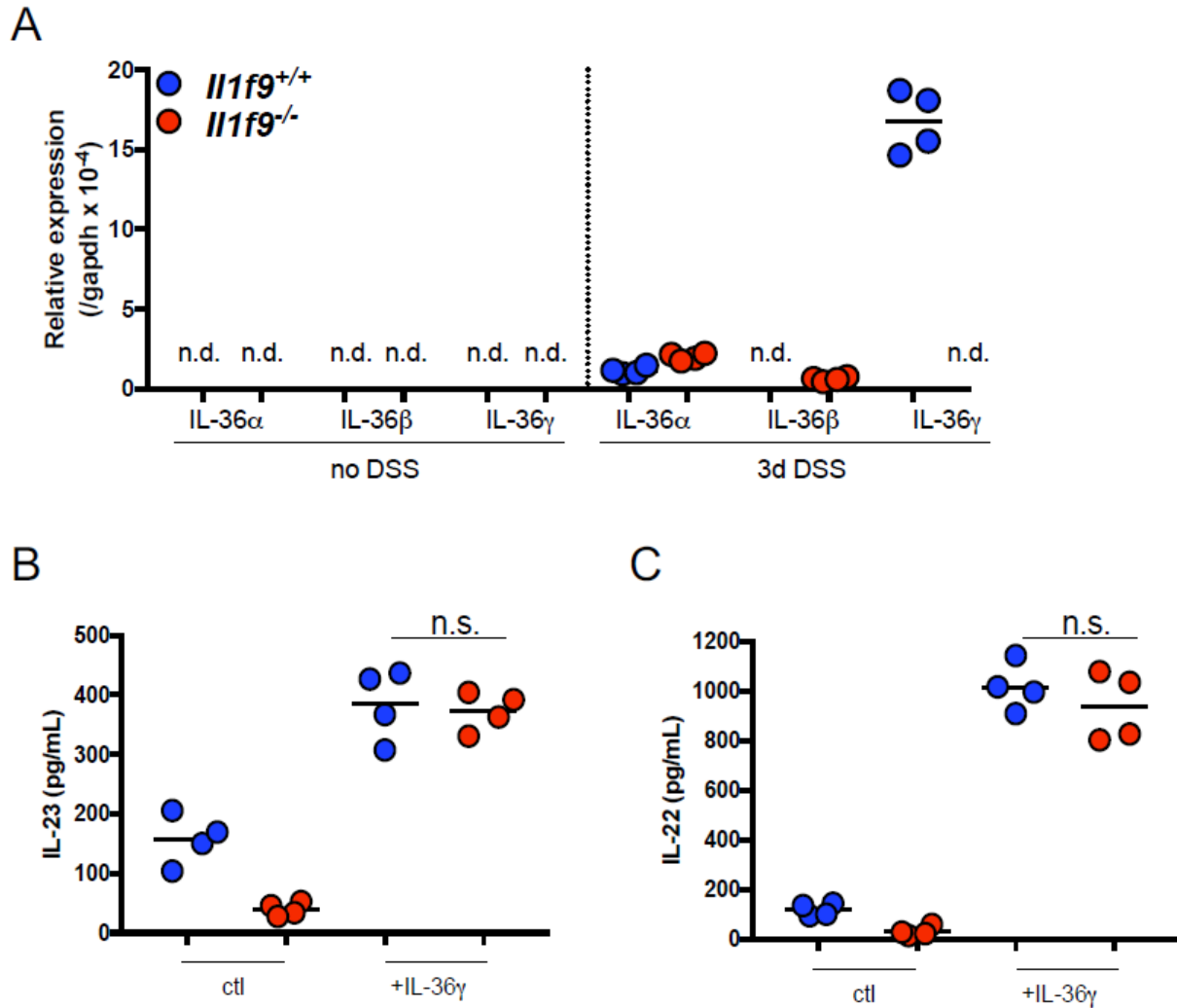


Figure 2.11 Analysis of *Il1f9*^{-/-} mice.

(A) IL-36 ligand mRNA expression in colonic tissues from 3-day DSS- treated *Il1f9*^{+/+} and *Il1f9*^{-/-} mice. (B) Colonic explants from control (no DSS) or 3-day DSS-treated (3d DSS) *Il1f9*^{+/+} and *Il1f9*^{-/-} mice were cultured for 60h in the presence or absence of IL-36 γ . Supernatants were analyzed for IL-23 (B) and IL-22 (C) by ELISA. n.d.= not detected. Data are representative of 2 independent experiments with 3-4 mice/group. All data are presented as mean \pm SEM; n.s.= not significant.

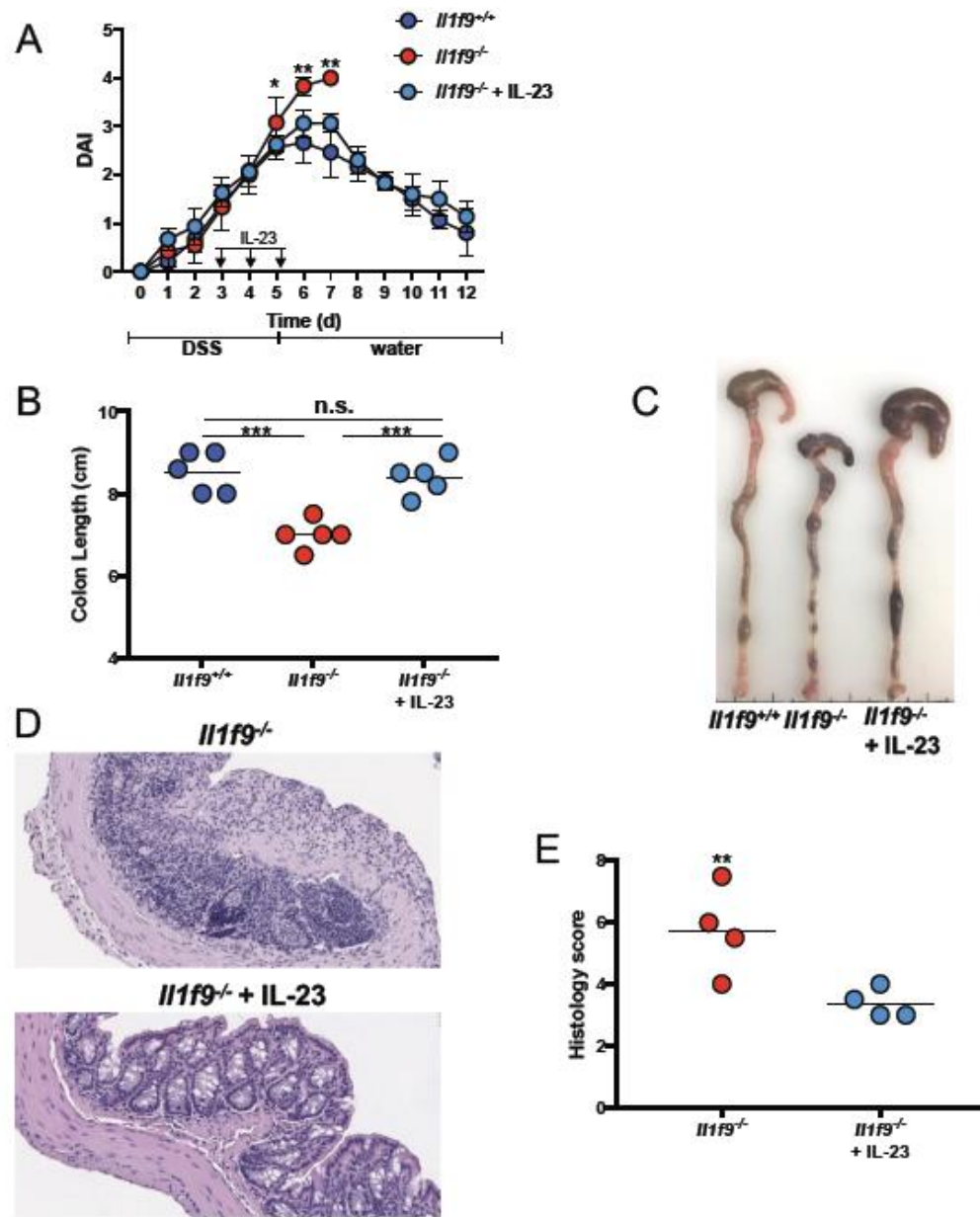


Figure 2.12 Systemic IL-23 administration induces resolution of DSS-induced colonic damage in $Il1f9^{-/-}$ mice.

(A) DAI of $Il1f9^{+/+}$ and $Il1f9^{-/-}$ mice treated with DSS for 5 days, followed by normal water for 7 days, in the presence or absence of IL-23. (B-C) Image and length of colons from mice treated as in (A). (D) H&E staining and (E) histology scoring of colon sections from mice treated as in (A). Data are representative of 3 independent experiments with 4-5 mice/group. All data are presented as mean \pm SEM; n.s. = not significant; * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$, one-way ANOVA with Tukey's multiple comparison test.

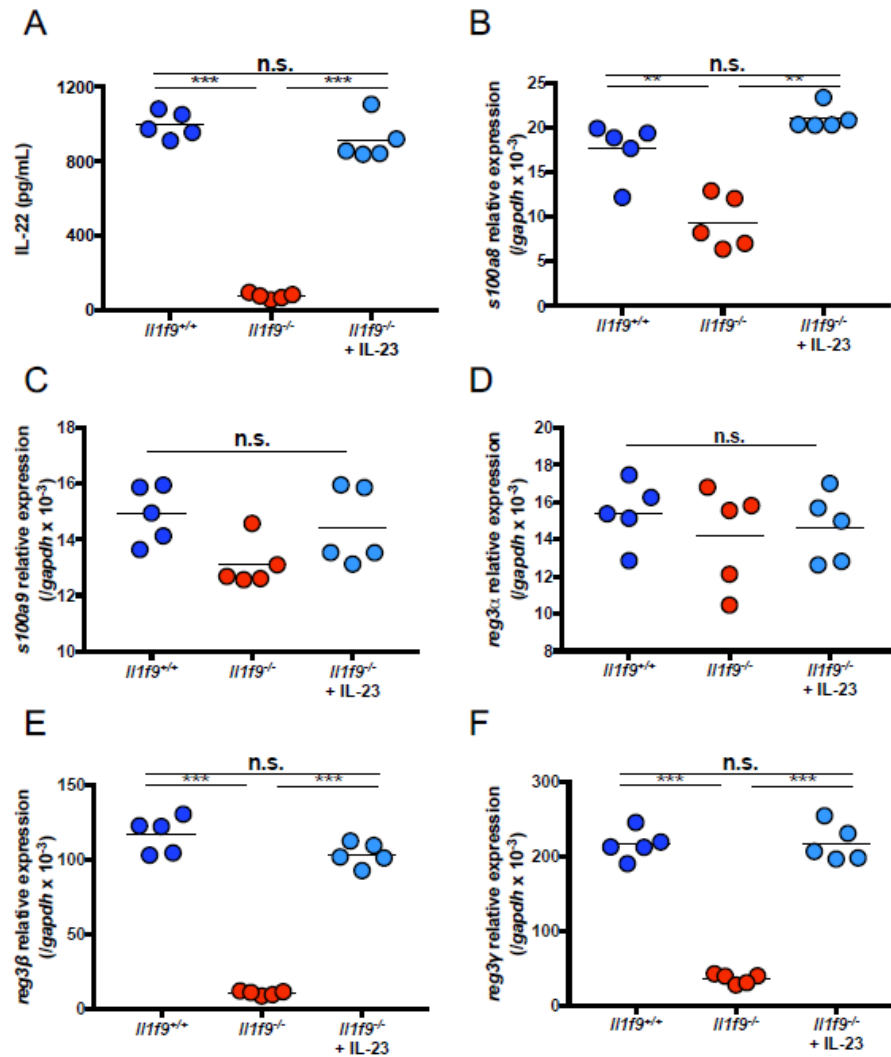


Figure 2.13 Systemic IL-23 administration induces IL-22 and AMPs in DSS-treated *Il1f9*^{-/-} mice.

(A) IL-22 protein expression in colons from *Il1f9*^{+/+} and *Il1f9*^{-/-} mice treated with DSS for 5 days in the presence or absence of IL-23. (B) S100A8 (C) S100A9 (D) Reg3α (E) Reg3β and (F) Reg3γ mRNA expression in colons isolated from mice as in (A). Data are representative of 2 independent experiments with 5-6 mice/group. All data are presented as mean ± SEM; n.s.= not significant; **P < 0.01, and ***P < 0.001, one-way ANOVA with Tukey's multiple comparison test

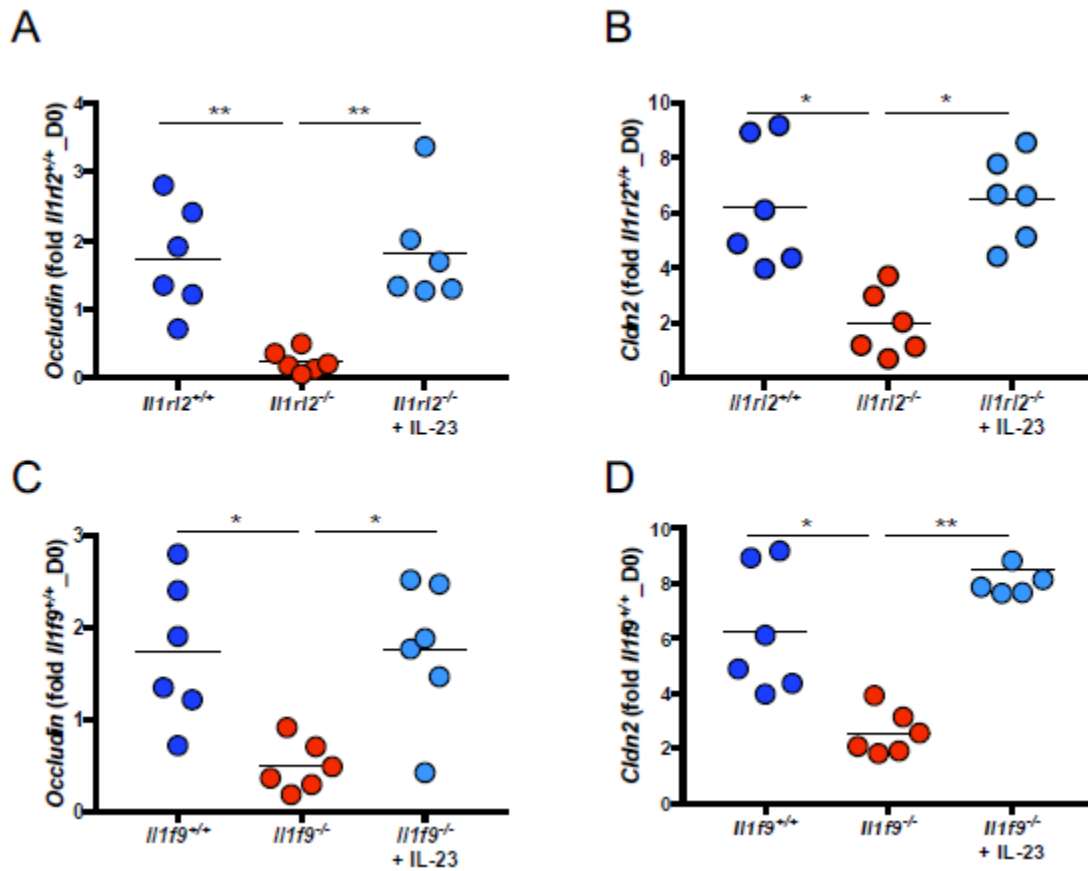


Figure 2.14 Systemic IL-23 administration induces expression of occludin and claudin 2 in DSS-treated *Il1rl2*^{-/-} and *Il1f9*^{-/-} mice.

(A) Occludin and (B) claudin 2 mRNA expression in colons from *Il1rl2*^{+/+} and *Il1rl2*^{-/-} mice treated with DSS for 5 days in the presence or absence of IL-23. (C) Occludin and (D) claudin 2 mRNA expression in colons from *Il1f9*^{+/+} and *Il1f9*^{-/-} mice treated with DSS for 5 days in the presence or absence of IL-23. Data are combined from 2 independent experiments with 3 mice/group. All data are presented as mean \pm SEM; * P < 0.05 and ** P < 0.01, one-way ANOVA with Tukey's multiple comparison.

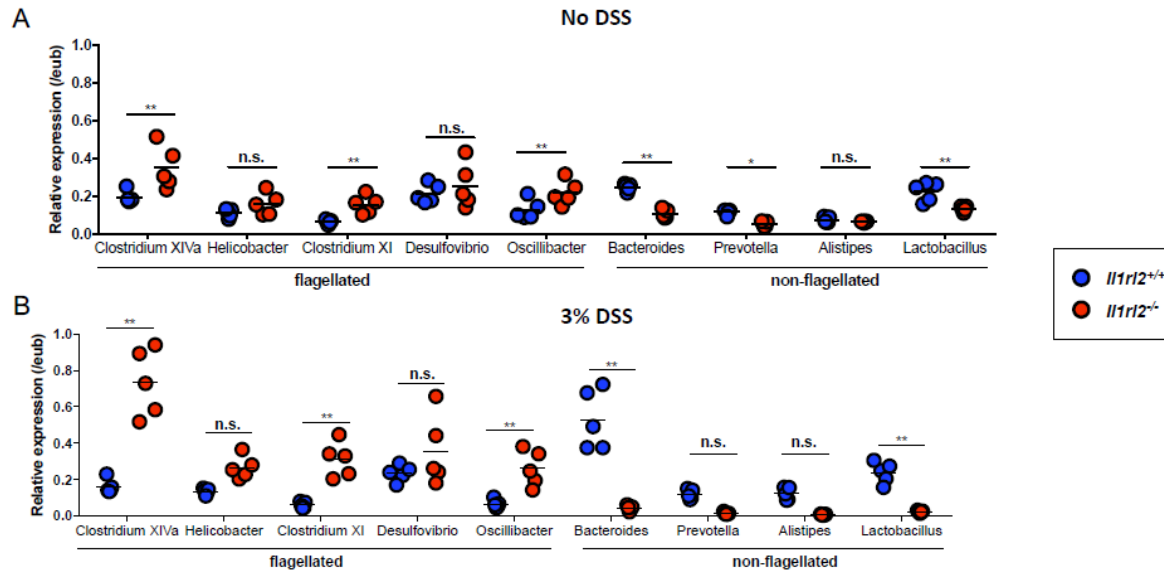


Figure 2.15 Fecal microbiota composition in steady-state and DSS-treated *Il1rl2*^{-/-} mice.

Real-time PCR analysis of flagellated and non-flagellated bacterial groups in the feces of *Il1rl2*^{+/+} mice and *Il1rl2*^{-/-} (A) before DSS treatment (no DSS) and (B) after DSS treatment. All data are presented as mean \pm SEM; * $P < 0.05$ and ** $P < 0.01$, one-way ANOVA with Tukey's multiple comparison test.

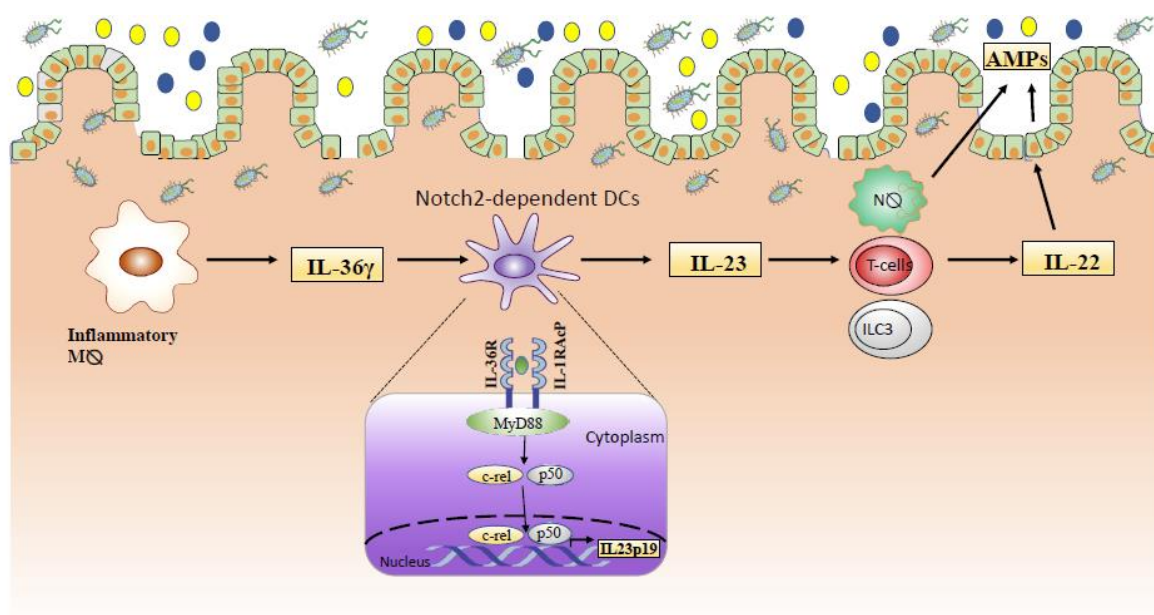


Figure 2.16 Schematic representation of the IL-36/IL-23/IL-22/AMPs axis.

3 CHAPTER 3: IL-36R SIGNALING INTEGRATES INNATE AND ADAPTIVE IMMUNE-MEDIATED PROTECTION AGAINST ENTEROPATHOGENIC BACTERIA

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3.1 Abstract

Infection with enteropathogenic bacteria is worldwide health problems and associated with a high mortality rate in developing countries. Efficient host protection against enteropathogenic bacterial infection is characterized by proper coordination responses between epithelial cells, innate, and adaptive immunity. In response to infection, the immune cells are activated to produce interleukin (IL)-22, which is involved in induces antimicrobial peptides to eliminate bacteria. The IL-36 cytokines are recently described members of IL-1 superfamily cytokines known to exert potent resolution of mucosal damages during acute intestinal inflammation via the induction of IL-22 and epithelial cell proliferation. IL-36R-deficient mice have reduced IL-22 expression; thus, these mice are more susceptible to acute intestinal inflammation and bacterial infection. Using a restricted mouse pathogen, *Citrobacter rodentium*, as a model to study intestinal bacterial infection, we demonstrated IL-36 signaling is a crucial component that helped linked epithelial cells, innate and adaptive immunity together to ward off intestinal bacterial infection. We showed that *Il1rl2*^{-/-} mice exhibiting decrease in AMPs, increase intestinal damage, and failed to resistance to bacterial colonization compared to wild-type control mice. This defective is associated with prominent downregulation of IL-23 and IL-6, two potent IL-22 inducers. Exogenous IL-23 administration during the early phase of bacterial infection mediated ILC3/IL-22 protection in *Il1rl2*^{-/-} mice. Whereas administration of IL-6 during the late phase of infection, mediated IL-

22 dependent CD4⁺ T cells protection. We also show the intracellular signaling cascade for IL-36 γ induces IL-6/IL-22 involves signaling through NF κ B-p65 subunit and the activation of aryl hydrocarbon receptor. Collectively, these data indicated that IL-36 signaling is providing critical host defense against intestinal bacterial infection.

3.2 Introduction

Attaching and effacing bacterial infection (A/E infection) of the gastrointestinal tract represents a significant cause of mortality worldwide and continues to pose a threat to global health. Human enteropathogenic *Escherichia coli* (EPEC) and enterohemorrhagic *E. coli* (EHEC) are the two common A/E bacterial infections and have been associated with a high infant mortality rate in developing nations. Up to date, much of our knowledge on how the host's response to A/E infections is derived from experimental studies with *Citrobacter rodentium*, a gram-negative mouse-restricted pathogen. *C. rodentium* colonizes the host's intestinal mucosal layer via the formation of attaching and effacing lesions that result in a breach of the intestinal epithelial barrier, leading to colitis via robust inflammatory responses [128-130].

Effective host protection against A/E bacterial infection is characterized by the combined responses of intestinal epithelial cells, innate and adaptive arms of the immune system. The intestinal epithelial cells produce antimicrobial peptides to control the pathogens [131]. Studies of *C. rodentium* infection in immune-deficient mouse strains have recognized the crucial role of innate lymphoid cells 3 (ILC3), and CD4⁺ T cells are essential components of the innate and adaptive immunity used for containing and eliminate bacterial [132-135]. Cytokines secrets from immune cells, including interleukin (IL)-22, IL-23, and IL-6, have an important role in host defense during *C. rodentium* infection. IL-22 plays a vital role in control the gut flora and pathogenic bacterial and is secreted by a heterogeneous population of immune cells. IL-22

control *C. rodentium* infection by stimulates the secretion of antimicrobial peptides in epithelial cells. Consequently, mice lacking this cytokine rapidly succumb to the disease [74, 131-133, 136-138].

The IL-1 family of cytokines is central to protective mucosal immunity in bacterial infection [139]. The IL-1 family of cytokines comprises seven agonistic cytokines and one anti-inflammatory member. IL-36 cytokines are a member of the larger IL-1 family and include three agonist proteins and one antagonist. IL-36 agonists bind to IL-36R complex and trigger immune responses. IL-36R is widely expressed on numerous cells, including murine dendritic cells, naïve CD4⁺ T-cells, epithelial cells of tissues such as the skin, intestine, and lung. Similar to their receptor, IL-36 ligands are expressed by a variety of cells dependent on the tissues and disease condition. In the murine model of acute intestine inflammation, IL-36 expressed by inflammatory monocytes and epithelial cells. IL-36 agonist mediates broad proinflammatory effects, including cytokines/chemokines production, DC maturation, and T-cell differentiation [34, 35, 66, 67]. IL-36 research has mainly focused on skin diseases; however, in the last decade, the role of IL-36 cytokines in intestine biology has emerged. Our group and others previously demonstrated that IL-36 cytokines undertake a central role in mediates the resolution of the intestinal mucosal damages in acute intestinal inflammation by facilitates epithelial cell proliferation and the production of IL-22, and antimicrobial peptides. IL-36R-deficient mice are more prone to DSS-induced acute intestinal inflammation and intestinal/lung bacterial infection [45, 47, 55, 56].

In this study, we tested the hypothesis that an IL-36R agonist, IL-36 γ , shape the host immunity in the murine intestines caused by *C. rodentium* infection. We found that IL-36 α and IL-36 γ are expressed in the large intestine during *C. rodentium* infection and that it mediates bacterial clearance in the intestines, innate inflammatory cells trafficking, cytokines/chemokines

expression. Moreover, we identify a critical role of IL-36 as a central component of innate and adaptive immune responses during *C. rodentium* infection. Specifically, our finding indicates in the early phase of infection, IL-36R signaling in promoting IL-23 release, which subsequently regulates the protective function of ILC3. During the late phase of infection, IL-36R signaling contributes to host defense by induces the expression of IL-6 and consequently directed the differentiation of naïve CD4⁺ T cells into IL-22 producing CD4⁺ T cell. Unlike IL-36R-sufficient mice, *Il1rl2*^{-/-} succumbed to *C. rodentium* infection due to the profound downregulation of IL-22 as compared to control mice. Administration of exogenous IL-23 in the early phase, or IL-6 in the late phase, protected *Il1rl2*^{-/-} mice from *C. rodentium* induced intestinal inflammation. Mechanistic experiments revealed that upon infected with *C. rodentium*, inflammatory secreted IL-36 γ and led to the activation of NF κ B in dendritic cells and subsequent induction of IL-23 and IL-6, which in turn promoted a protection of ILC-3 and CD4⁺ T cells mediated IL-22 response.

3.3 Materials and Methods

3.3.1 Mice

The following mice were purchased from The Jackson Laboratory: WT C57BL/6 (B6 WT), B6.129P-*Nfkb1*^{tm1Bal/J} (*p50*^{-/-}), B6.129P2(SJL)-*Myd88*^{tm1.1Defr/J} (*myd88*^{-/-}), B6.SJL-*Ptprc*^a*Pepc*^b/BoyJ (CD45.1), B6.129S6-*Tbx21*^{tm1Glm/J} (*tbx21*^{-/-}). *Il1rl2*^{-/-} (IL-36R^{-/-}) mice on the C57BL/6 background (backcrossed more than nine generations) were originally provided by Amgen. To generate *Il1f9*^{-/-} (IL-36 γ ^{-/-}) mice, sperm from IL-36 γ ^{-/-} mice was obtained from Knockout Mouse Project repository (University of California, Davis), and heterozygous *Il1f9*^{+/-} founder mice were generated by Mouse Transgenic and Gene Target Core facility at Emory University. *Il1f9*^{+/-} mice were then bred to generate *Il1f9*^{-/-} mice on the C56BL/6 background (backcrossed more than nine generation). *Ahr*^{-/-} mice kindly provided by Dr. Andrew Gewirtz (Georgia State University).

Unless otherwise stated mice were used at 4-6 weeks of age and were carried out using age and gender matched groups. Animal studies were approved by the Institutional Animal Care and Use Committee of Georgia State University.

3.3.2 *Citrobacter rodentium* strain and infection

C. rodentium strain ICC180 (derived from DBS100) was originally generated by Dr. Gad Frankel and Dr. Fiouxsie Wiles (Imperial College London) and was generously gifted by Dr. Casey Weaver (The University of Alabama, Birmingham). Mice were inoculated with $5-6 \times 10^9$ colony forming per unit (CFU) in a volume of 200 μ l PBS via gastric gavage and monitored for survival rate, body weight changes and bacterial colonization for clinical signs of disease.

3.3.3 *Bioluminescence Imaging*

Mice were anesthetized with isoflurane and imaging with an IVIS-100 system and Living Image Software (Xenogen, Inc.). Baseline images were collected prior bacterial inoculation and whole images were taken at binning of 4 over 3-10 mins at the indicates times during infection.

3.3.4 *Gene expression analysis of RT² profiler PCR array*

Gene expression analysis of whole colonic tissues infected with *C. rodentium* at day 4 and 8 were assess using RT² profiler PCR array (Qiagen) as previously described. Briefly, at indicated time point, mice infected with *C. rodentium* were euthanized and whole colonic tissues were homogenized and isolated RNA by using RNeasy mini kit (Qiagen) and the cDNA was generated using RT² First Strand Kit (Qiagen) followed by genomic isolation. Profile of genes expression from generated cDNA were construct according to manufactured protocol.

3.3.5 *CD4⁺ T cell differentiation*

FACs-sorted CD4⁺CD25⁻ T cells were co-culture in the presence of FACs-sorted CD45⁺MHCII⁺CD11c⁺ dendritic cells (DCs) for 72-80 hours in the presence or absence of

100ng/mL IL-36 γ . In some experiments, monoclonal antibodies and/or pharmaceutical inhibitors were added in the culture as indicated per experiments. Unless otherwise stated, monoclonal antibodies were purchased from R&D and inhibitors were purchased from Cayman chemicals.

3.3.6 ELISA

IL-22 and IL-6 secretion was measured in cell-free culture supernatant using IL-22 ELISA kit (R&D system) and IL-6 ELISA kit (eBiosciences) according to the manufacturer's protocol.

3.3.7 *In Vivo Administration of IL-23 and IL-6*

Recombinant mouse IL-23 and IL-6 was purchased from R&D system. All administration of recombinant cytokines was performed via intraperitoneal injection. For IL-23 administration, *Il1rl2*^{-/-} mice received either PBS or 0.5 μ g of IL-23 at day 0 and 2 post infection. For IL-6 administration, *Il1rl2*^{-/-} mice received either PBS or 1 μ g of IL-6 at day 4, 6 and 8 post infection.

3.3.8 *Bone marrow-derived dendritic cells (BMDCs) and bone marrow-derived macrophages (BMDMs)*

BMDCs and BMDMs were generated from 6 to 15-week-old WT, *crel*^{-/-}, *p50*^{-/-}, *myd88*^{-/-} mice. Bone marrow cells were harvest from femurs of mice and cultured in RPMI-1640 supplemented with fetal bovine serum, 50 μ β -mercaptoethanol, and penicillin/streptomycin. Depend of BMDCs and BMDMs, 20ng/mL of recombinant murine granulocyte macrophage colony-stimulating factor (GM-CSF), or 20ng/mL of macrophage colony-stimulating factor (M-CSF) (Peprotech, NJ) were added to cells. The generated BMDCs and BMDMs were harvested and 2x10⁵ cell/well were seeded to the 96 well plates for further studies. For *in vivo* study of the induction of IL-36 γ , BMDMs were stimulated with head-killed *C. rodentium* for 24 hours. For the *in vivo* study of how IL-36 γ induces IL-6, WT BMDCs were stimulated \pm IL-36 γ (100ng/mL) and

± NF-κB inhibitors: c-Rel inhibitor IT-603 (Calibiochem, CA), p50/or p65 inhibitor peptide (Novus biological, CO).

3.3.9 *Quantitative real-time PCR*

Total RNA was isolated from mice colonic tissues using the Qiagen RNeasy Mini Kit according to manufacturer's protocol. cDNA was generated with Superscript First Strand Synthesis kit according to manufacturer's protocol. Q-PCR was performed with SYBR green (BioRad, CA) using a StepOnePlus PCR system (Applied Biosystem, CA) and *gapdh* were used to normalizes gene expression. Primer used in the experiments are list below:

Il22

Fwd 5'-CAGGAGGTGGTGCCTTTCCT-3'

Rev 5'-TGGTCGTCACCGCTGATGT-3'

Il23

Fwd 5'-GCTGTGCCTAGGAGTAGCAG-3'

Rev 5'-TGGCTGTTGTCCTTGAGTCC-3'

Il1f6

Fwd 5'-TAGTGGGTGTAGTTCTGTAGTGTGC-3'

Rev 5'-GTTCGTCTCAAGAGTGTCCAGATAT-3'

Il1f8

Fwd 5'-ACAAAAAGCCTTTCTGTTCTATCAT-3'

Rev 5'-CCATGTTGGATTACTTCTCAGACT-3'

Il1f9

Fwd 5'-AGAGTAACCCCAGTCAGCGTG-3'

Rev 5'-AGGGTGGTGGTACAAATCCAA-3'

Il1rl2

Fwd 5'-AAACACCTAGCAAAAGCCCAG-3'

Rev 5'-AGACTGCCCGATTTTCCTATG-3'

Reg3a

Fwd 5'-GGCACCGAGCCCAATG-3'

Rev 5'-GGATTTCTCTCCCATGCAAAGT-3'

Reg3b

Fwd 5'-ATGCTGCTCTCCTGCCTGATG-3'

Rev 5'-CTAATGCGTGCGGAGGGTATATTC-3'

Reg3g

Fwd 5'-TTCCTGTCCTCCATGATCAAAA-3'

Rev 5'-CATCCACCTCTGTTGGGTTCA-3'

S100a8

Fwd 5'-TGAGTGTCTCAGTTTGTGCAG-3'

Rev 5'-TGTGAGATGCCACACCCACTTT-3'

S100a9

Fwd 5'-CAAATGGTGGAAGCACAGTTGGCA-3'

Rev 5'-TTGTGTCCAGGTCCTCCATGATGT-3'

Gapdh

Fwd 5'-CAAATGGTGGAAGCACAGTTGGCA-3'

Rev 5'-TTGTGTCCAGGTCCTCCATGATGT-3'

Dll1

Fwd 5'- GCT GGA AGT AGA TGA GTG TGC TC -3'

Rev 5'- CAC AGA CCT TGC CAT AGA AGC C -3'

Dll2

Fwd 5'- CCA GCA CTG GAT GCC TTT TAC C -3'

Rev 5'- ACC TCA CAT CGA AGC CCG TAG A -3'

Dll4

Fwd 5'- GGG TCC AGT TAT GCC TGC GAA T -3'

Rev 5'- TTC GGC TTG GAC CTC TGT TCA G -3'

Jagged1

Fwd 5'- GAG ATA TAC CGC ACC CCT TCA G -3'

Rev 5'- ACC ACC TGC GAA GTG ATT GAC -3'

Jagged2

Fwd 5'- TCC TCA TCT GGA GTG GTG TCA T -3'

Rev 5'- CGT CGT CAT TCC CTT TCA GTT C -3'

3.3.10 Chromatin Immunoprecipitation assay (ChiP)

BMDC were cultured with \pm IL-36 γ (100ng/mL) for 6 hours. ChiP assay were performed with EZ-ChiP kit (EMD Millipore 17371) as previous described (PNAS paper). Briefly, chromatin was obtained from BMDCs after fixation with formaldehyde and fragmented by sonication. One tenth of each fragmented sample were saved prior to immunoprecipitation for input control. The fragmented sample was immunoprecipitated with control antibody or anti p50 (clone SC-8418), anti c-rel (clone sc-6955) (Santa Cruz Biotechnology, CA), or anti p65 (clone K310) (Abcam, MA). Input sample and DNA recovered from the immunoprecipitation were analyzed by qPCR.

3.3.11 Histology

Colon tissues were fixed in 10% neutral formalin buffer. Paraffin embedding, sectioning and hematoxylin/eosin staining, and slide scanning were performed at Histowiz, Inc. (New York).

3.3.12 Statistical analysis

All statistical analyses were done with GraphPad Prism software, version 8.0. One-way ANOVA and Tukey's multiple comparison or Student's *t* test were used to determine significance (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$, ns not significance).

3.4 Results

3.4.1 IL-36 γ and IL-36 α are induced in the large intestine during *C. rodentium* infection.

In order to establish the role of IL-36 in response to intestinal bacterial infection, we assessed whether IL-36 agonist ligands are expressed in *C. rodentium* infection. Wild-type mice were inoculating with $5\text{-}6 \times 10^9$ CFU of *C. rodentium* and the kinetic expression of *Il1f6* (IL-36 α), *Il1f8* (IL-36 β), and *Il1f9* (IL-36 γ) were measured in the large intestine of infected mice over twelve days. Between the three agonists, the IL-36 γ transcript was express at the earliest, beginning at day one, and peaking at day six then steadily declining after eight-day post-infection (Figure 3.7 A). IL-36 α were transcribed on day two with the level of expression steadily increasing over a twelve days period post-infection (Figure 3.7 B). In contrast to IL-36 α and IL-36 γ , no IL-36 β was not detected in the *C. rodentium* infected large intestine (Figure 3.7 C). In this study, we have opted to focus on IL-36 γ as it is the preferred IL-36 ligand expressed during experimental intestinal inflammation in mice, and all the IL-36 agonists had been shown to have similar functions.

IL-36 γ expression has been observed in several cell types ranging from the hematopoietic to the non-hematopoietic compartment in different murine models of inflammation, because of this, we analyzed several immune cells population for IL-36 γ RNA at day six post-infection.

Among immune cell populations, CD11b⁺Ly6C⁺ inflammatory monocytes were the most significant producer of IL-36 γ (Figure 3.7 D). Moreover, *in vivo* stimulating bone-marrow derived macrophages (BMDMs) with heat-killed *C. rodentium* resulted in robust induction of IL-36 γ (Figure 3.7 E). Collectively, these results suggested the contribution of IL-36 signaling in promoting intestinal immunity to *C. rodentium*.

3.4.2 IL-36 signaling promotes pathogen clearance and colonization resistance against *C. rodentium* infection.

Recently, IL-36R signaling has been shown to have prominent roles in host protection against oral fungal and lung bacterial infection [140-142]. To examine the significance of IL-36R signaling caused by enteropathogenic intestinal bacterial infection, we infected *Il1rl2*^{+/+} and *Il1rl2*^{-/-} mice with 5x10⁹ – 6x10⁹ colony-forming unit (CFU) of a bioluminescence *C. rodentium* and monitored weight loss, bacterial colonization, and survival rate of mice. Consistent with previous study [43], bioluminescence imaging of the mice's bodies revealed that *Il1rl2*^{+/+} mice were able to survive the infection by completely clearing out the bacteria by eighteen days post-infection. In stark contrast, *Il1rl2*^{-/-} mice failed to control bacterial colonization, experienced a slow bacterial clearance rate (Figure 3.1 A and Figure 3.8 A), and succumbed to infection starting at day ten and leading to a 40% mortality by day eighteen post-infection (Figure 3.1 B). *Il1rl2*^{-/-} mice also experienced a substantial drop in bodyweight (Figure 3.1 C) and had significantly higher bacterial shedding in feces (Figure 3.1 D). Bioluminescence imaging the mice's organs ten days post-infection revealed that while the bacterial load was higher in the large intestine and the caecum of the *Il1rl2*^{-/-} mice, the *C. rodentium* did not spread to spleen, liver and mesenteric lymph nodes (Figure 3.8B). Furthermore, while IL-36R-deficient mice shown to have higher bacterial counts in large intestine and caecum, their peripheral organs have the similar number of bacteria compared

to the control mice (Figure 3.8, C to F). These data are suggested that while IL-36 signaling is crucial to control bacterial colonization and accelerate bacterial clearance, this pathway is dispensable for control the dissemination of bacteria.

3.4.3 IL-36R-deficiency results in diminished IL-22, IL-23, and IL-6 expression in the large intestine during *C. rodentium* infection.

We and other groups had demonstrated that mice with altered in IL-36 signaling experienced compromised mucosal healing and were increasingly susceptible to bacterial infection. For the potential mechanisms of IL-36R signaling conferred protection to host against intestinal bacterial infection, our search began with a PCR array on the total colonic tissues isolated from *Il1rl2*^{+/+} and *Il1rl2*^{-/-} mice at early and late phase of infection, day 4 and 8, respectively (Figure 3.2 A). PCR array analysis revealed that in the early phase of infection IL-22 and IL-23 expression was eighteenfold and ten-fold, respectively, higher in *Il1rl2*^{+/+} mice compared to *Il1rl2*^{-/-} mice (Fig. 3.2, B and D). This indicates that similar to DSS induced acute intestinal inflammation, during the early phase of bacterial infection, the impaired of IL-22 expression in *Il1rl2*^{-/-} mice is correlated with the deficiency of IL-23. During the late phase of infection, the analysis showed that the both IL-22 and IL-6 expression was tenfold higher in *Il1rl2*^{+/+} mice compared to *Il1rl2*^{-/-} mice (Figure 3.2, C and E). ELISA analysis of colonic tissues for IL-22, IL-23 and IL-6 protein expression during twelve days post-infection revealed that IL-23, IL-6 and IL-22 was significantly higher in colonic tissue isolated from *Il1rl2*^{+/+} mice compared to *Il1rl2*^{-/-} mice (Figure 3.2, F to H). Previous studies indicated that IL-22 is the crucial component in host protection against intestinal enteric bacteria, and that, while the early wave of IL-22 production is dependent on IL-23, the second wave of IL-22 is dependent on IL-6. These previous findings, in conjunction with our data,

implies that IL-36 signaling potentially participates in both the innate and adaptive arms of the immune systems in response to intestinal bacterial infection.

Of note, IL-36 signaling also implicating in control the recruitment of inflammatory innate immune cells during bacterial infection. Thus, we questioned whether abnormalities in the IL-36 pathway could affect the recruitment of immune cells to the large intestine during bacterial infection. Analysis of the innate immune subset at the early phase (day 4) and the late phase (day 8) of infection revealed no difference in the percentage of inflammatory monocytes (CD11b⁺LyC⁺) and neutrophils (CD11b⁺Ly6G⁺) during the early phase of infection, however, these two subsets are significantly elevated during the late phase (Figure 3.9, A to C). The elevation of both inflammatory monocytes and neutrophils in *Il1rl2*^{+/+} mice corresponds to the increased gene expression of neutrophil chemoattractant *cxcl1* and *cxcl2* (Figure 3.9, D and E), as well as monocyte chemoattractant, *cxcl3* and *cxcl5* (Figure 3.9, F and G).

3.4.4 Early administration of IL-23 rescues *Il1rl2*^{-/-} mice from *C. rodentium* infection.

Our group demonstrated that IL-36 cytokines induce IL-22 is IL-23-dependent and the administration of IL-23 can rescue *Il1rl2*^{-/-} mice from DSS-induced acute colonic damage. As IL-23 is essential for host protection in the early phase of bacterial infection, we postulated that the injection of IL-23 could confer protection to *Il1rl2*^{-/-} mice against *C. rodentium* infection. *C. rodentium* infected *Il1rl2*^{-/-} mice received either PBS or rIL-23 (0.5ug) at day 0 and 2 post-infection, and mice were monitored for bacterial clearance, change in body weight, and survival (Figure 3.3 A). Systemic administration of IL-23 to *C. rodentium* infected *Il1rl2*^{-/-} mice was able to accelerate bacterial clearance, improved survival rate, and body weight (Figure 3.3, B to E). Furthermore, the histology of infected *Il1rl2*^{-/-} mice was similar to those observed in *Il1rl2*^{+/+} mice (Figure 3.3 F and G). Antimicrobial peptides (AMPs) induced by IL-22, including S100A8,

S100A9 and RegIII family, are crucial for the host's defense against bacterial infection. Thus, we investigated whether *Il1r2*^{-/-} mice exhibit decreased AMP expression during *C. rodentium* infection. Due to their inability to induce IL-22, consequently *Il1r2*^{-/-} mice experienced a significantly diminished expression of AMPs compared to that of wild-type mice at four days post-infection. This defect appeared to be nearly reversible by delivering exogenous IL-23 (Figure 3.3, H to K).

We next determined which immune cell populations in the IL-36 pathway produced IL-22 in response to exogenous IL-23. *Il1r2*^{-/-} mice infected with *C. rodentium* were treated with rIL-23 at days 0 and 2 post-infection. They then received either α CD90 antibody to deplete T-cell and ILC3 populations or α CD4 to deplete CD4⁺T-cells. As expected, the *Il1r2*^{-/-} mice treated with IL-23 eliminated the bacteria and recovered while the mice treated with α CD90 failed to control bacterial colonization. At day 20, 60% of the mice treated with α CD90 succumbed to the disease. We noticed that, remarkably, the mice which had received α CD4 could only control bacterial colonization the first eight-day post-infection; as a result, these mice experienced a rapid decrease in body weight and an increase in bacterial presence (Figure 3.3, L to O). In addition, FACs data showed that exogenous rIL-23 failed to induce CD4⁺ T cells to produce IL-22 at four days post infection (Figure 3.10, A and B). These data indicated that while IL-36 acts on ILC3 during the early phase of infection; in the late phase of infection, IL-36 exerts its influences on CD4⁺ T cells to confer host protection.

3.4.5 Administration of IL-6 during late phase of C. rodentium infection is associated with accelerated bacterial clearance and restoration of IL-22 production.

We have shown that, during early bacterial infection, administration of exogenous IL-23 can rescue host; however, this effect is interrupted in the absence of CD4⁺ T-cells. We also

observed that in the late phase of infection, *Il1rl2*^{-/-} mice experienced reduced expression of IL-6 and IL-22. Since IL-6 is a well-known potent inducer for CD4⁺ T cell to produce IL-22. Therefore, we hypothesized that we could rescue *Il1rl2*-deficient mice during the late phase of bacterial infection by administration of exogenous IL-6. *C. rodentium* infected *Il1rl2*^{-/-} mice received either PBS or IL-6 (1 μ g) at days 4, 6, and 8 and mice were monitored for 20 days (Figure 3.4 A). Interestingly, while *C. rodentium* infected *Il1rl2*^{-/-} mice failed to control the bacterial colonization in the first few days; these mice were able to accelerate bacterial clearance quickly upon receiving exogenous IL-6 (Figure 3.4 B). By day 20 post-infection, bacterial shedding in feces of *Il1rl2*^{-/-} infected mice treated with IL-6 were comparable to that of *Il1rl2*^{+/+} infected mice (Figure 3.4 C). In addition, while 20% of *Il1rl2*^{-/-} mice perished after 20-day post infection, administration of IL-6 to *Il1rl2*^{-/-} mice increased their survival rate to 100% (Figure 3.4 D), and these mice also lost less body weight (Figure 3.4 E) and had histology scores identical to that of wild-type mice (Figure 3.4, F and G).

IL-22 and IL-6 are both critical for host protection against bacterial infection due to their ability to stimulate the production of appropriate antimicrobial peptides [74, 131, 137]. We next examined if *Il1rl2*^{-/-} mice were defective of IL-22 and AMPs expression following bacterial infection and if this could be reversed by IL-6 administration. Following 10-day post-infection, *Il1rl2*^{+/+} mice expressed a high level of IL-22 and antimicrobial peptides including, RegIII β , RegIII γ , S100A9. Consistent with their inability to produce IL-22 and IL-6, *Il1rl2*^{-/-} mice expressed lower levels of AMPs in response to bacterial infection. This effect could be overturned with IL-6 administration (Figure 3.4, H to K). However, the protective effect of IL-6/IL-22 signaling pathway was rendered completely ineffective when using antibody-mediated depletion of CD4⁺ T-cell in *Il1rl2*^{-/-} mice (Figure 3.4, B to K). Collectively, these results demonstrate that

delivery of IL-6 to *Il1rl2*^{-/-} mice is sufficient to restore IL-22 and AMPs expression, and rescues mice from intestinal bacterial infection.

3.4.6 *IL-36 signaling through AhR induces IL-22 from CD4⁺ T cells in IL-6-dependent manner.*

We established that IL-36 signaling affects both the innate and adaptive cells of the immune system to provide host protection against bacterial infections. In addition, we demonstrated that depletion of CD4⁺ T cells abrogates protection in the late phase of the disease. Next, we sought to explore the contribution of the IL-36 signaling axis on naïve CD4⁺ T-cell differentiation. We first investigated whether IL-36γ could regulate IL-22 secretion *in vitro*. Sorted naïve CD4⁺CD25⁻ T cells and CD11c⁺ dendritic cells from spleens of wild-type mice were either cultured alone or co-cultured and stimulated with IL-36γ in the presence of αCD3. In the presence of IL-36γ, T cells and lone-cultured DCs failed to induce IL-22; however, co-cultured T/DC cells produced a robust expression of IL-22 (Figure 3.5 A). Because both naïve CD4⁺ T cells and DCs had been showed to express IL-36R, we questioned whether IL-36γ was using CD4⁺ T cells or DCs to stimulate the induction of IL-22. We employed a co-culture system whereby DCs and naïve CD4⁺ T cells were isolated from *Il1rl2*^{+/+} and *Il1rl2*^{-/-} mice. Interestingly, the expression of IL-36R by DCs was found to be indispensable for the induction of IL-22 in the T/DC co-culture (Figure 3.5 B and Figure 3.11, A and B). AhR and T-box transcription factor (T-bet) have been implicated as transcription factors for IL-22-producing CD4⁺ T cells. Therefore, we next examined whether IL-36γ signals through AHR and T-bet to differentiate naïve CD4⁺ T cells into IL-22-producing CD4⁺ T cells. We performed T/DC co-cultures where the cells were isolated from either *ahr*^{+/+} or *ahr*^{-/-} mice. In the presence of IL-36γ, T/DC from *ahr*^{+/+} mice have a robust induction of IL-22 and this effect is completely abolished when using T/DC from *ahr*^{-/-} mice (Figure 3.5 C).

Furthermore, using a well-known pharmaceutical inhibitor CH-223191, to block AHR activation in T/DC co-culture sorted from *ahr*^{+/+} mice and stimulated with IL-36 γ also nullified the ability of IL-36 to induce IL-22 expression from T cells (Figure 3.11 C). Of note, CH-223191 did not interfere with cells proliferation in the culture as the level IL-2 expression of CH223191-treated cells are comparable with the untreated cells (Fig. 3.11 D). We next explored whether the absence of T-box transcription factor can impact the ability of IL-36 γ to induce IL-22 from T cells. Co-cultured T/DCs isolated from *t-bet*-deficient mice that had been stimulated with IL-36 γ resulted in a modest decrease in IL-22; however, this induction of IL-22 was entirely annulled in the presence of AhR pharmaceutical inhibitors (Figure 3.11 E). These data demonstrated that AHR is a crucial component in the IL-36/CD4⁺/IL-22 pathway.

Gene expression analysis of colonic tissue from *Il1rl2*^{+/+} and *Il1rl2*^{-/-} mice during the late phase of *C. rodentium* infection revealed that *Il1rl2*^{+/+} mice expressed a much higher amount of IL-6 and IL-22. In addition, *in vivo* administration of exogenous IL-6 can restore IL-22 secretion in *Il1rl2*^{-/-} mice (Figure 3.4, H to J). Thus, we investigated whether IL-36 γ was acting to promote the expression of IL-22 from CD4⁺ T cells via the induction of autocrine/paracrine signaling, including IL-6, which is a known factor for Th17/Th22 differentiation. We observed that T cell and DCs isolated from the spleen of wild-type mice were co-cultured and stimulated with IL-36 γ was able to prompt a strong IL-22 expression (approximately fourfold) higher compared with unstimulated control. We next tested whether the blockade of IL-6 can have an impact on IL-22 expression. Antibody-mediated neutralization of IL-6 was substantially decreased (approximately 60%) the effect of IL-36 γ on T/DC cells to induce IL-22 (Figure 3.5 E and Figure 3.12 A). Of note, using IL-23p19 and IL-23p40 antibodies mediated-blockade of IL-23 or T/DCs isolated from *Il12b*^{-/-} mice, only had a modest effect in IL-22 expression when stimulated cells with IL-36 γ

(Figure 3.12 A and B). Also, neutralization of α IL-1 β , α IL-4, α TGF- β , α IFN- γ , and α TNF- α did not affect IL-36 γ mediated IL-22 expression (Figure 3.12 A).

3.4.7 *IL-36 γ induces IL-6 expression in DCs via MyD88 and NF- κ B-p65 signaling.*

It is well-known that IL-6 is produced by antigen-presenting cells [143, 144]. Indeed, bone marrow-derived dendritic (BMDCs) responded to recombinant IL-36 γ to producing IL-6 secretion (Figure 3.5 F). Giving that IL-6 is an essential factor for IL-36 γ to differentiate naïve CD4⁺ T-cell into IL-22-CD4⁺ producing T-cells, we sought to understand the signaling cascade involved in IL-6/IL-22 induction in response to IL-36 γ . We begin our investigation with MyD88 adaptor protein because this protein is a crucial adaptor for signaling pathways downstream of IL-1 family members [145]. We generated BMDCs from *my88*^{+/+} and *myd88*^{-/-} mice and cultured them with IL-36 γ . In the presence of IL-36 γ , *my88*^{+/+} BMDCs induced a more robust expression of IL-6 (approximately six-fold) compared to *my88*^{-/-} BMDCs (Figure 3.13 A). Previous studies showed that upon interacting with its receptor, IL-36 ligands trigger an intracellular cascade leading to the activation of nuclear factor kappa B (NF- κ B) to stimulate immune responses [34, 48, 49, 56]. We then probed the role of NF- κ B in IL-36 γ -induced IL-6 in dendritic cells by using a small molecules inhibitor of NF- κ B. BMDCs generated from wild-type mice treated with the NF- κ B inhibitor, BAY 11-7082, virtually obliterated IL-36 γ mediated IL-6 expression. We next investigated which subunits of the NF- κ B were involved in IL-36 γ induced IL-6 expression. BMDCs from WT mice responded to IL-36 γ stimulation and induced a robust secretion of IL-6. This effect was diminished approximately 70% in the presence of the p65 inhibitor peptide, but not in the presence of p50 or c-rel inhibitors (Figure 3.5 G). Likewise, following stimulation with IL-36 γ , we observed no reduction of IL-6 from BMDCs generated from *p50*^{-/-} and *crel*^{-/-} mice (Figure 3.13 B and C). ChIP assay was performed to determined p50, c-rel and p65 binding to IL-6 promoter in BMDCs treated

with IL-36 γ . As shown in Figure 3.5 H, there was a significant increase in p65 binding to IL-6 promoter in response to treatment of BMDCs with IL-36 γ for 6 hours. Together, these findings demonstrate that MyD88 and subunit NF- κ B are part of a signaling cascade downstream of IL-36R that is involved in IL-6 expression in DCs.

3.4.8 IL-36 signaling induces IL-22 production in CD4⁺ T cells is partially dependent on Notch ligands.

Since *in vitro* neutralization of IL-6 did not completely inhibit the ability of IL-36 γ to induce IL-22 in the T/DCs co-culture, we hypothesized that IL-36 γ could potentially signal through other pathways to promote CD4⁺ T-cell production of IL-22. Recent studies have reported that either (1) signaling through the Notch pathway or (2) the degradation of tryptophan by the enzyme indoleamine 2,3 dioxygenase-1 (IDO1) to kynurenine could promote CD4⁺ T cells to express IL-22 [4, 5]. Hence, we examined whether these two pathways could be involved in IL-36 γ 's ability to differentiate naïve CD4⁺ T-cell into IL-22-producing CD4⁺T cells. To investigate the role of the kynurenine pathway, we treated T/DC cells with 1-methyltryptophan (1-MT), a pharmaceutical compound, that block IDO1 from degrade tryptophan to kynurenines. We observed that in the presence of IL-36 γ , 1-MT treated cells shown no detectable decrease in IL-22 from T/DC co-cultures (Figure 3.14 A). In addition, treated cell with 1-MT do not affect cell proliferation or cell's ability to induce cytokines as IL-2, IFN-g, and IL-17A secretion level from 1-MT treated cells are comparable with nontreated cells (Figure 3.14 B to D). Next, we sought to define the role of Notch signaling in the IL-36R/IL-22 pathway. Cells were treated with GSI, a pan-Notch inhibitor. The secretion of IL-22 in T/DC was reduced by approximately 40% in the presence of GSI and IL-36 γ (Figure 3.6 A).

Having found that IL-36 γ can use the Notch pathway to induce IL-22 from CD4⁺ T cells, we next sought to determine which notch ligands were involved in the IL36 γ /Notch/IL-22 cascade. The canonical notch signaling pathway has five ligands: delta-like protein 1 (*dll1*), delta-like protein 3 (*dll3*), delta-like protein 4 (*dll4*), jagged 1 (*jag1*) and jagged 2 (*jag2*); these ligands are responsible for the majority of the Notch signaling effect. BMDCs were generated from wild type mice and stimulated with IL-36 γ . FACs analysis and qPCR analysis revealed that IL-36 γ mediated the upregulation of *dll1* and *dll4* among the five Notch ligands (Figure 3.6 B and C and Figure 3.6 G to I) and found no detectable changes for *dll3*, *jag1*, and *jag2* (Figure 3.6 D to F). We then assessed whether a DLL1 and DLL4 blockade could have an impact on IL-36 γ induced IL-22 expression in co-cultured T/DCs. While antibody-mediated neutralization of DLL1 showed a minimal effect on IL-22, blockade of DLL4 drastically diminished IL-22 (approximately 30%). Consistently, neutralization of both IL-6 and DLL-1/4 completely terminated the effect of IL-36 γ induces IL-22 in T/DC co-culture (Figure 3. 6 J).

Base on the data presented on this report, we proposed a following model for the role of IL-36 signaling in host protection against intestinal inflammation: upon *C. rodentium* infection, inflammatory monocytes release IL-36 agonists and the IL-36 bind its cognate receptor complex on dendritic cells and triggers a signaling cascade mediating the secretion of IL-23, IL-6 and notch ligands (DLL1/4). While IL-23 binds its receptor on ILC3 to propagate the production of IL-22 and subsequently AMPs to promote protection during early phase of infection; IL-6 and notch ligands bind to their respective receptors on CD4⁺ T cells to activate AHR and ultimately differentiate naïve CD4⁺ T cells into IL-22-producing CD4⁺ T cells to promote protection for host during late phase of infection (Figure 3.15).

3.5 Discussion

In this study we provide evidence demonstrating that signaling via IL-36R critical for control of a model enteric bacterial pathogen – *Citrobacter rodentium*. Mice deficient in IL-36R exhibited decreases in AMPs, increase intestinal damage, and impaired resistance to bacterial colonization. These defects were associated with diminished IL-23 and IL-6 – the respective early and late inducers IL-22. Consistent with these data, exogenous IL-23 administration during the early phase of bacterial infection mediated ILC3/IL-22 protection in *Il1rl2*^{-/-} mice, whereas administration of IL-6 during the late phase of infection, mediated IL-22-dependent CD4⁺ T cell protection. Our data also demonstrate that IL-36 γ induces IL-6/IL-22 via NF κ B-p65 and aryl hydrocarbon receptor-dependent mechanisms. Overall, these data highlight a fundamental contribution of signaling via the IL-36/IL-36R axis to the early (innate) and late (adaptive) control of enteric bacterial infection and colitis.

Previous data have established that type 3 innate lymphoid cell (ILC3) production of IL-22 is instrumental in driving early host protection against *Citrobacter rodentium* [74]. Mice deficient in IL-22, cannot control *Citrobacter rodentium* expansion and rapidly succumb to infection. In addition to early IL-22 production by ILC3, late expression of IL-22 by CD4⁺ T cells is also critical for protection against *Citrobacter rodentium*. Interestingly, while early IL-22 production by ILC3s is IL-23 dependent, late IL-22 production by CD4⁺ T cells was shown to be IL-6 dependent and IL-23 independent [131, 146]. Therefore, unique cytokines induce IL-22 during distinct windows of enteric bacterial infection. Our data presented here demonstrate that signaling via IL-36R is an important upstream inducer of both early IL-22 production by ILC3s as well as late IL-22 production by CD4⁺ T cells.

We have previously reported that IL-36 ligands – particularly IL-36 γ , were induced in the DSS model of colitis and IL-36 γ was similarly induced in the intestine following *Citrobacter rodentium* infection. In the absence of signaling via IL-36R, the induction of IL-23 and IL-6 were both dramatically reduced, as was early and late IL-22 expression. Importantly, IL-36R-deficient mice could be rescued from uncontrolled *Citrobacter rodentium* infection by exogenous supplementation of IL-23 early or IL-6 late. Consistent with the DSS model of colitis, signaling via IL-36R affords critical host protective effects through the induction of IL-22 and AMPs. Thus, the inflammatory role of IL-36 is instrumental to barrier protection from acute damage as well as transient bacterial infection. This contrasts with the pathogenic role of IL-36R signaling during chronic intestinal inflammation [46], such as observed in the oxazolone model of colitis where IL-36 induces potent T_H9 expansion and T_{reg} inhibition to enhance disease [54].

Our study indicates that therapeutic targeting of IL-36R in inflammatory processes, such as inflammatory bowel disease (IBD), may be optimized by a clear understanding of the dual roles this pathway plays in acute and chronic conditions. It is attractive to envisage blockage of IL-36R while concomitantly delivering IL-22 and/or AMPs in order to ameliorate pathogenic inflammation while promoting barrier recovery. This concept may not only apply to the IL-36 pathway, but also other IL-1 family members, and even other inflammatory cytokines such as IL-17A that have both inflammatory and barrier protective functions. Additionally, our data may provide insight into reasons for the lack of long-lasting beneficial effects of some biological therapies used to treat ulcerative colitis and Crohn's disease.

3.6 Figures and Figures Legends

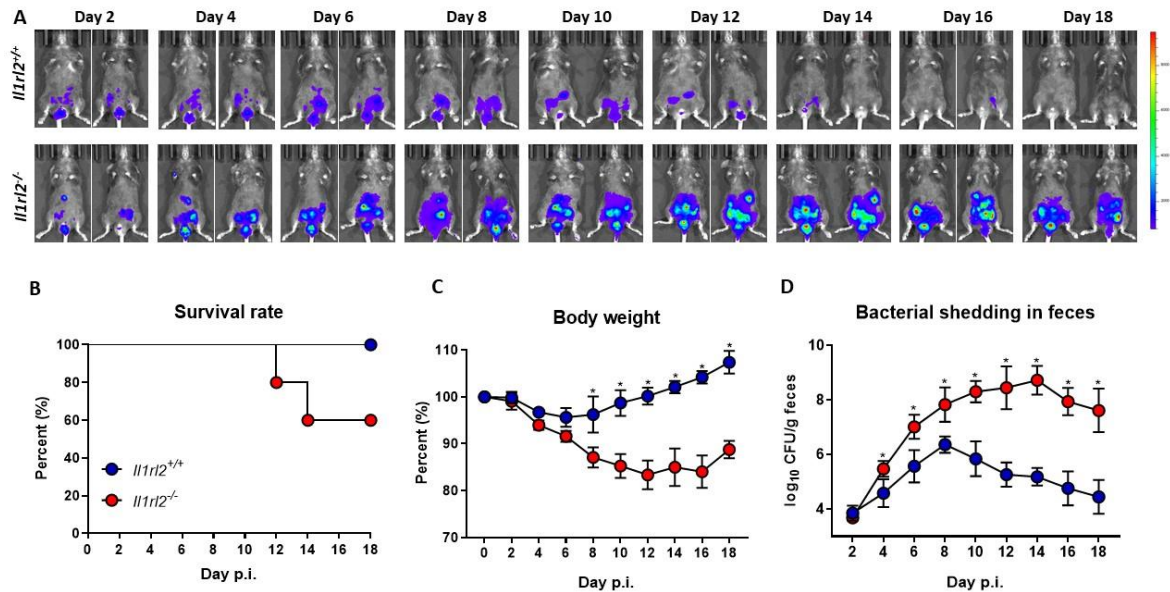


Figure 3.1 IL-36 signaling promotes pathogen clearance and colonization resistance against *C. rodentium* infection.

Four to six-week-old *Il1rl2*^{+/+} and *Il1rl2*^{-/-} mice infected with bioluminescence *C. rodentium* (5×10^9 CFU) by gastric gavage. **(A)** Serial whole-body imaging of *Il1rl2*^{+/+} and *Il1rl2*^{-/-} mice at indicated time points. Images are representative of 2 independent experiments. **(B to D)** **(B)** Survival rate; **(C)** Average body weight changes; **(D)** Bacterial shedding in feces of infected *Il1rl2*^{+/+} and *Il1rl2*^{-/-} mice at indicated time points. Data are representative of three independent experiments with 5 mice per group. All data presented as mean \pm SEM (Multiple t tests - one per row, correct for multiple comparisons using the Holm-Sidak method. * $P < 0.5$)

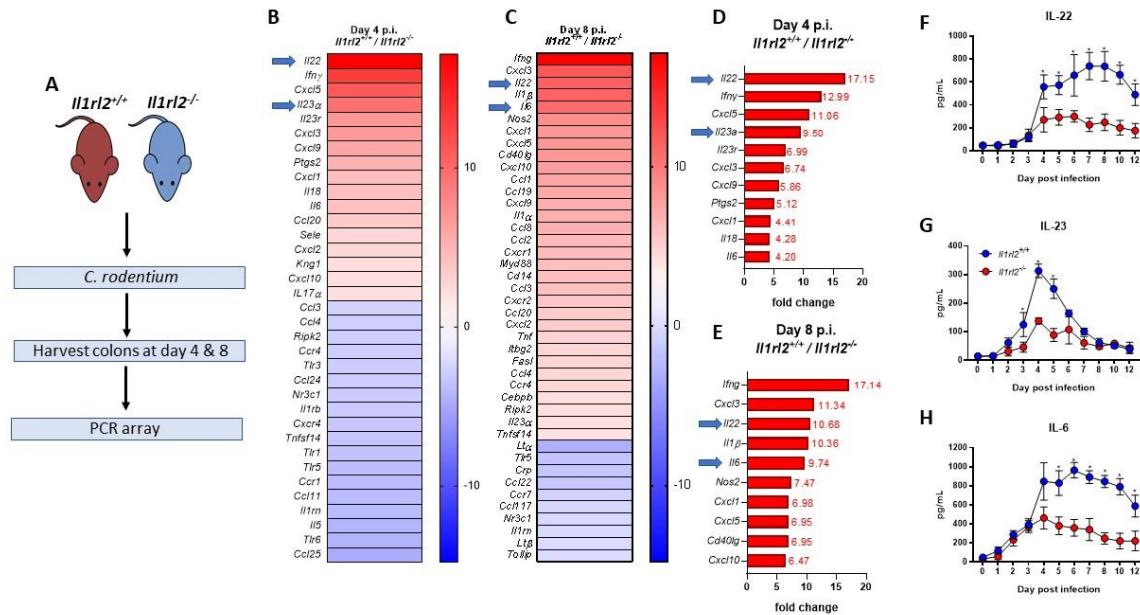


Figure 3.2 IL-36R-deficient results in diminished IL-22, IL-23, and IL-6 expression in the large intestine during *C. rodentium* infection.

(A) Experimental schematic of intestinal bacterial infection by oral infected *Il1rl2*^{+/+} and *Il1rl2*^{-/-} mice with 5-6x10⁹ CFU of *C. rodentium*. (B and C) PCR array gene expression analyses from large intestine tissues isolated at indicated time points post infection of *Il1rl2*^{+/+} and *Il1rl2*^{-/-} mice. (D and E) The top ten significantly expressed genes with highest upregulation were obtained from PCR array analysis between *C. rodentium* infected *Il1rl2*^{+/+} and *Il1rl2*^{-/-} mice at day four post infection (D) and day 8 post infection (E). (F to H) The time course of (F) IL-22 (G) IL-23 (H) IL-6 protein expression from colonic tissues isolated from infected mice. Data are representative of two independent experiments with 4-5 mice per group. All data are presented as mean ± SEM. *P < 0.5.

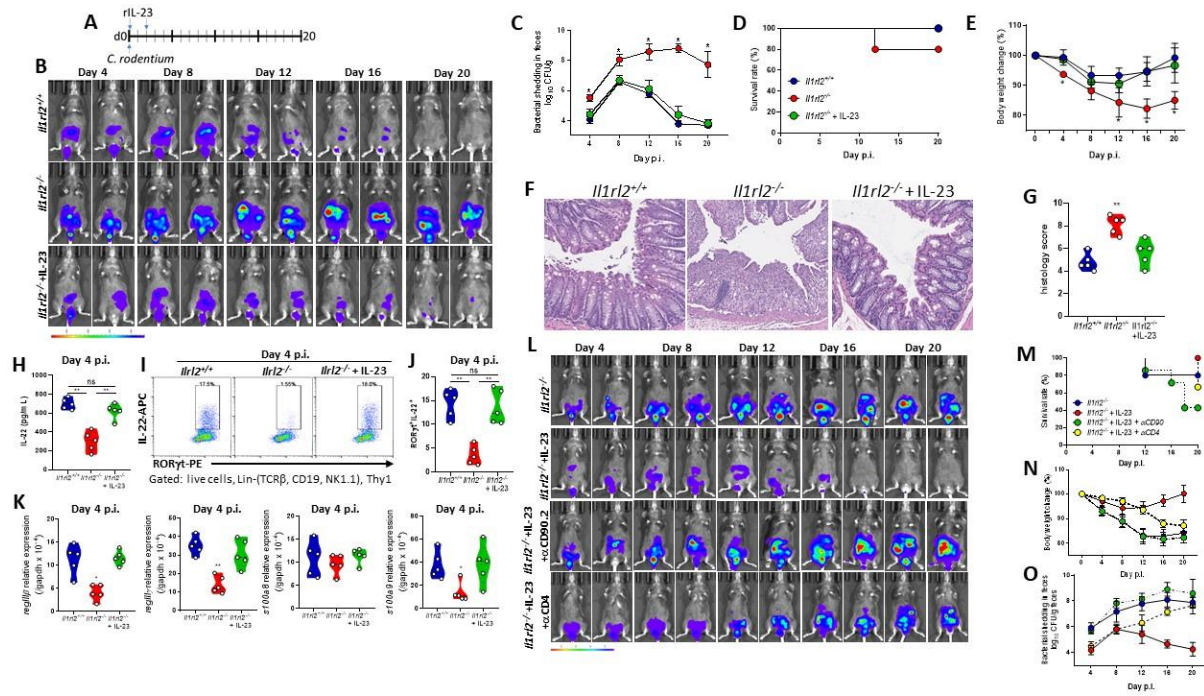


Figure 3.3 Early administration of exogenous IL-23 rescues *Il1rl2*^{-/-} mice from *C. rodentium* infection.

(A) Experimental schematic of *C. rodentium* infection by gastric gavage *Il1rl2*^{+/+} and *Il1rl2*^{-/-} mice with 5-6x10⁹ CFU of *C. rodentium*, in the presence or absence of IL-23. (B) Serial whole-body imaging of infected mice at indicated time points. Images are representative of 2 independent experiments with at least 5 mice/group. (C to E) (C) average body weight change; (D) survival rate; (E) bacterial shedding in feces of infected mice at indicated time points. (F and G) (F) The H&E staining and histology scoring (G) of colon sections from infected mice as in (A) are shown. (H) IL-22 protein expression in the colon at ten days post *C. rodentium* infection from *Il1rl2*^{+/+} and *Il1rl2*^{-/-} mice. (I) Colonic lamina propria cells of *C. rodentium* infected mice at four days post infection were isolated analyzed by FACS for expression of intracellular IL-22 by Thy1⁺RORγt⁺ gated cells. (J) FACS frequency data of Thy1⁺RORγt⁺IL-22⁺ gated cells of infected mice generated as in (I). (K) Antimicrobial peptides mRNA expression in colon from infected mice at four days post infection. (L) Serial whole-body imaging of *C. rodentium* infected *Il1rl2*^{-/-} mice in the presence or absence of IL-23 and neutralization antibodies, αCD90 or αCD4 at indicated. Images are representative of 2 independent experiments. (M to O) (M) survival rate; (N) average body weight changes; (O) bacterial shedding in feces of infected mice as in (L) at indicated time points. Data are representative of three independent experiments with 5 mice per group. All data presented as mean ± SEM (one-way ANOVA with Tukey's multiple comparison test. **P* < 0.5; ***P* < 0.05; ****P* < 0.001; ns, not significant)

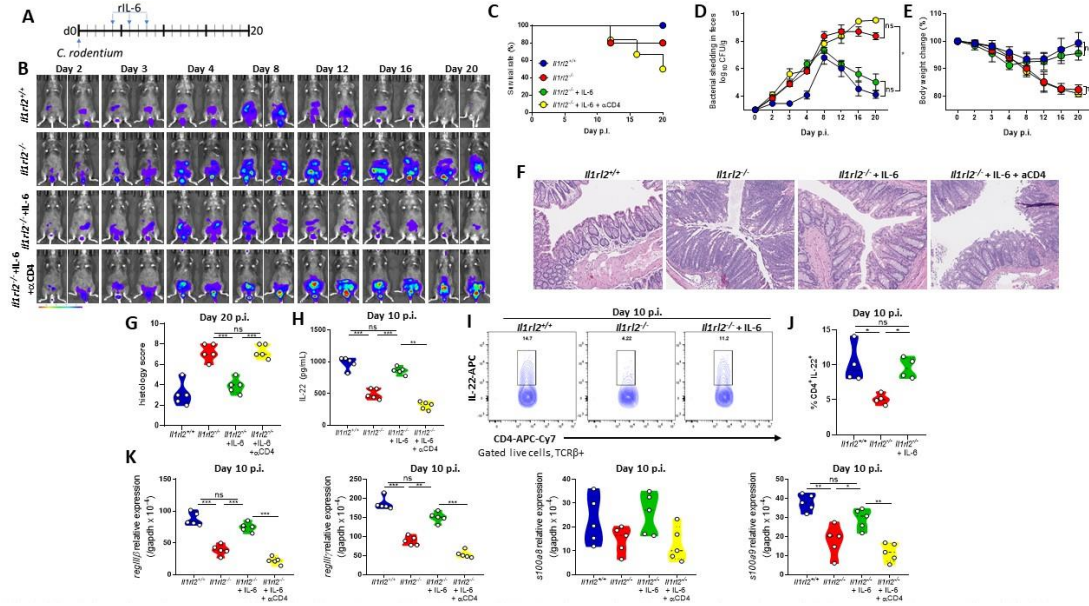


Figure 3.4 Administration of exogenous IL-6 during late phase of *C. rodentium* infection is associated with accelerate bacterial clearance and restoration of IL-22.

(A) Experimental schematic of intestinal bacterial infection by gastric gavage *Il1rl2*^{+/+} and *Il1rl2*^{-/-} mice with 5-6x10⁹ CFU of *C. rodentium*, in the presence or absence of recombinant IL-6 and/or αCD4. (B) Serial whole-body imaging of infected mice at indicated time points. Images are representative of 2 independent experiments with at least 5 mice/group. (C to E) (C) survival rate; (D) average body weight changes; (E) bacterial shedding in feces of infected mice at indicated time points. (F and G) (F) The H&E staining, and (G) histology scoring of colon sections from infected mice as in (A) are shown. (H) IL-22 protein expression in colons from infected mice at ten days post infection. (I) Colonic lamina propria cells of *C. rodentium* infected mice as shown in (A) were isolated on day 10 post infection and analyzed by FACs for expression of intracellular IL-22 by TCRβ⁺CD4⁺ gated cells. (J) FACs frequency data from TCRβ⁺CD4⁺IL-22⁺ gated cells of *C. rodentium* infected mice generated base on (I). (K) Antimicrobial peptides mRNA expression in colon from infected mice at ten days post infection. Data are representative of three independent experiments with 5 mice per group. All data presented as mean ± SEM (one-way ANOVA with Tukey's multiple comparison test. *P < 0.5; **P < 0.05; ***P < 0.001; ns, not significant)

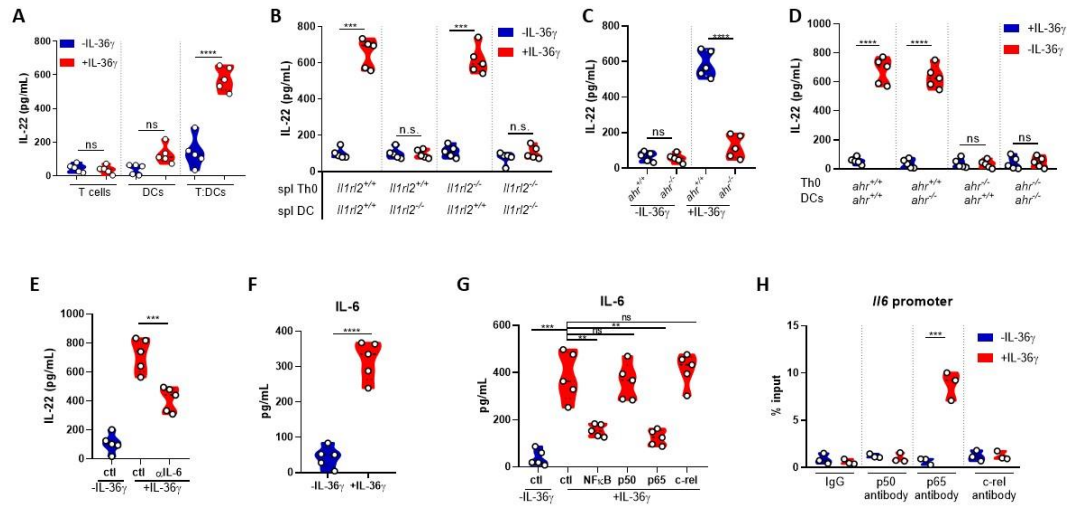


Figure 3.5 IL-36 signaling through an aryl hydrocarbon receptor induces IL-22 from CD4⁺ T cells is IL-6 -dependent.

(A) DCs and CD4⁺T cells from spleen of WT mice were FACs sorted and were cultured either alone or co-cultured for 72h in the presence or absence of IL-36γ. Supernatant were analyzed for IL-22 by ELISA. (B) FACs sorted naïve CD4⁺T cells and DCs were co-cultured using indicated cell from *Il1rl2*^{+/+} and/or *Il1rl2*^{-/-} mice with ± IL-36γ for 72h. IL-22 protein in supernatant was determined by ELISA. (C) IL-22 protein expression by FACs sorted co-culture DCs and naïve CD4⁺ T cells from *ahr*^{+/+} or *ahr*^{-/-} mice in the presence of IL-36γ for 72h. (D) FACs sorted naïve CD4⁺T cells and DCs were co-cultured using indicated cell from *ahr*^{+/+} and/or *ahr*^{-/-} mice with ± IL-36γ for 72h. IL-22 protein in supernatant was determined by ELISA. (E) FACs sorted DCs and CD4⁺ T cells from WT mice were co-cultured and stimulated with IL-36γ and αIL-6 antibody for 72hrs. IL-22 protein was assessed by ELISA. (F and G) (F) BMDCs were generated from WT mice and cultured in the presence or absence of IL-36γ for 24h, and IL-6 was assessed by ELISA. (F) Some cultures were pretreated with NFκB inhibitor, or c-Rel inhibitor, or p50 inhibitor, or p65 inhibitor or with vehicle control for 1hrs. (G) ChIP assays for p50, p65, and c-Rel binding to *Il6* promoter in BMDCs treated with ± IL-36γ for 8h. Data are representative of three independent experiments with 4-5 replicates. All data presented as mean ± SEM (one-way ANOVA with Tukey's multiple comparison test. **P* < 0.05, ***P* < 0.01, ****P* < 0.001; ns, not significant).

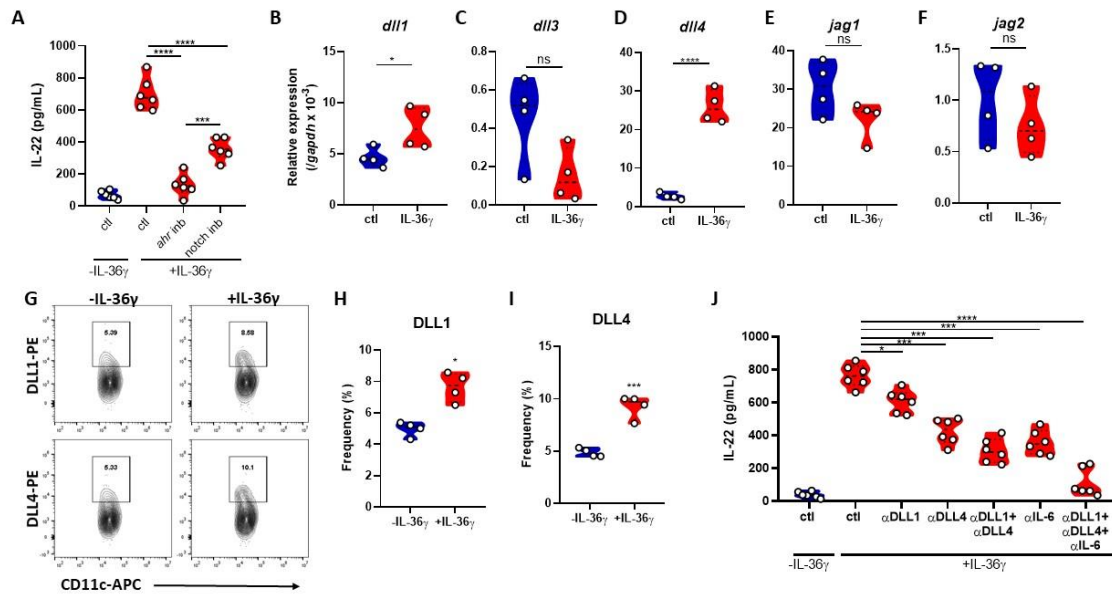


Figure 3.6 IL-36 signaling induces IL-22 from CD4 $^{+}$ T cells is dependent on Notch ligands.

(A) FACS sorted DCs and CD4 $^{+}$ T cells from wild-type mice were co-cultured and stimulated with IL-36 γ with the presence/absence of AHR or notch inhibitor for 72h. IL-22 protein expression were assessed by ELISA. (B to F) mRNA expression for Notch ligands from wild-type BMDCs stimulated with IL-36 γ for 6h. (B) *dll1* (C) *dll2* (D) *dll3* (E) *jag1* (F) *jag2*. (G to I) DLL1 and DLL4 expression by sorted wild type DCs cultured with \pm IL-36 γ for 16h. (G) Representative FACS plots. (H) the frequencies of DLL1 and (I) the frequencies of DLL4 among totals DCs. (J) FACS sorted DCs and naive CD4 $^{+}$ T cells from wild-type mice were co-cultured and stimulated with IL-36 γ supplemented with indicated monoclonal antibodies for 72h. IL-22 protein were assessed by ELISA. Data are representative of three independent experiments with 4-5 replicates. All data presented as mean \pm SEM (one-way ANOVA with Tukey's multiple comparison test. * P < 0.05, *** P < 0.001, **** P < 0.0001; ns, not significant)

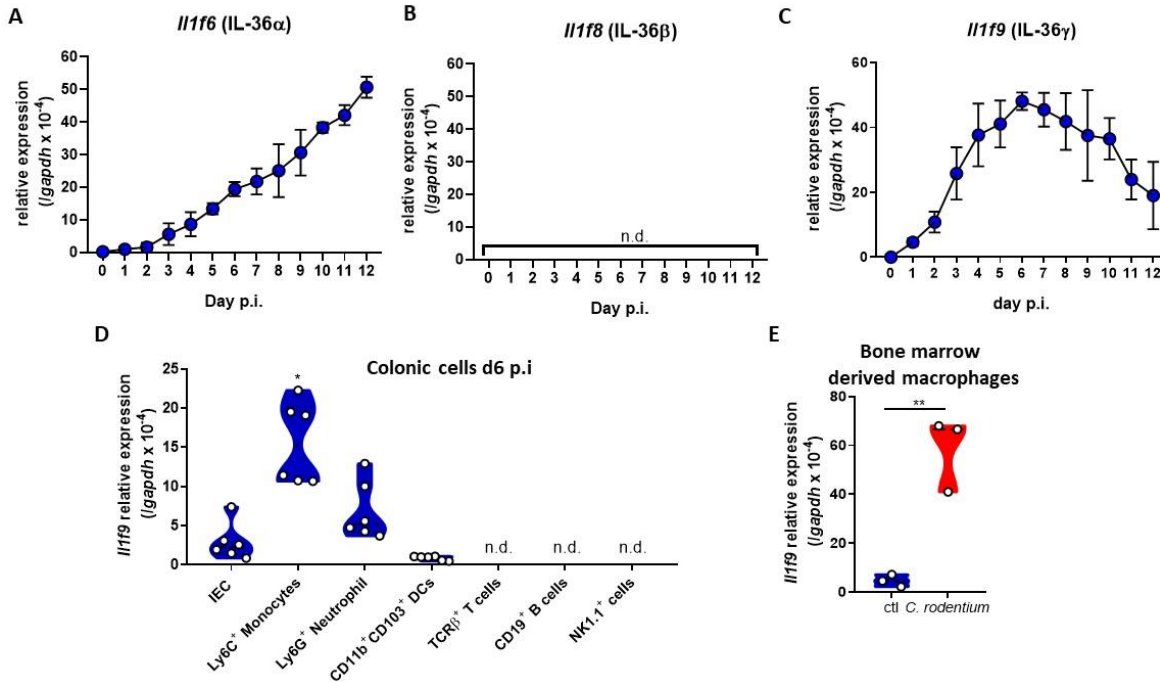


Figure 3.7 IL-36 γ and IL-36 α , but not IL-36 β , are induced in the large intestine during *C. rodentium* infection.

(A to C) Time course analysis *Il1f6* (A), *Il1f8* (B), and *Il1f9* (B) mRNA expression in the large intestine from *Il1rl2*^{+/+} mice infected with $5\text{-}6 \times 10^9$ CFU *C. rodentium*. (D) *Il1f9* mRNA expression from several large intestine immune cells at six days post-*C. rodentium* infection in *Il1rl2*^{+/+} mice. Data are representative of two independent experiments with 4-6 mice per group. (E) BMDCs were generated from *Il1rl2*^{+/+} mice and cultured with heat-killed *C. rodentium* for 6 hours, and IL-36 γ mRNA expression was assessed by real-time qPCR. Data are representative of two independent experiments with 3-4 replicates. All data presented as mean \pm SEM (one-way ANOVA with Tukey's multiple comparison test. * $P < 0.5$; ** $P < 0.05$).

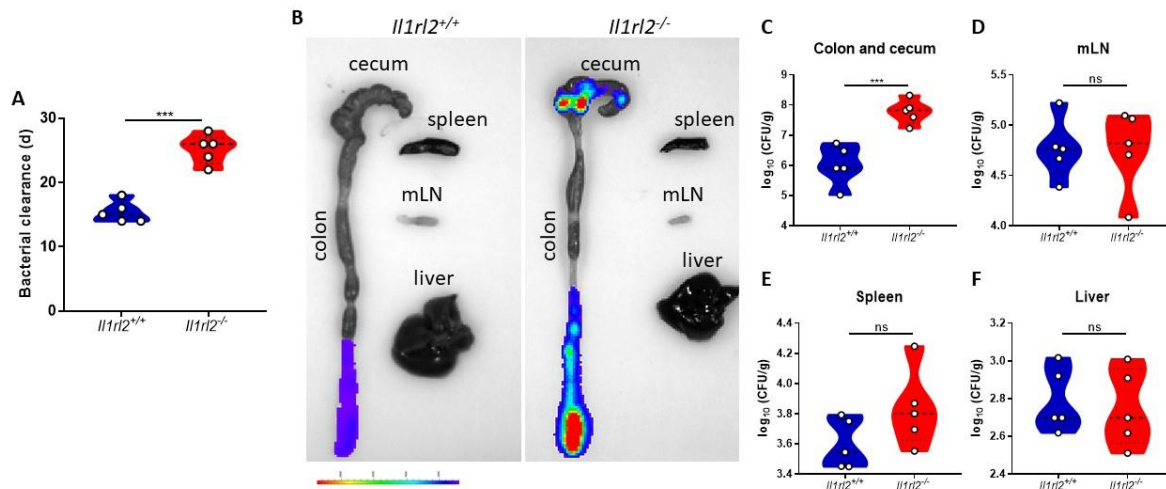


Figure 3.8 IL-36 signaling is required to accelerate bacterial clearance but is not necessary for controlling systemic dissemination of bacteria.

Four to six-week-old *Il1rl2*^{+/+} and *Il1rl2*^{-/-} mice infected with bioluminescence *C. rodentium* (5×10^9 CFU) by gastric gavage. **(A)** Bacterial clearance time of *Il1rl2*^{+/+} and *Il1rl2*^{-/-} mice infected with bioluminescence *C. rodentium* evaluated by calculating bacterial shedding in feces of mice at indicated timepoint. **(B)** Bioluminescence images of several organs from *Il1rl2*^{+/+} and *Il1rl2*^{-/-} mice ten days post infection with 5×10^9 CFU of *C. rodentium*. **(C to F)** Bacterial counts in colon and cecum **(C)**, mesenteric lymph nodes **(D)**, spleen **(E)**, and liver **(F)** of *Il1rl2*^{+/+} and *Il1rl2*^{-/-} mice infected with *C. rodentium* as in **(B)**. Data are representative of three two independent experiments with 4-5 mice per group. All data presented as mean \pm SEM (one-way ANOVA with Tukey's multiple comparison test. *** $P < 0.001$; ns, not significant).

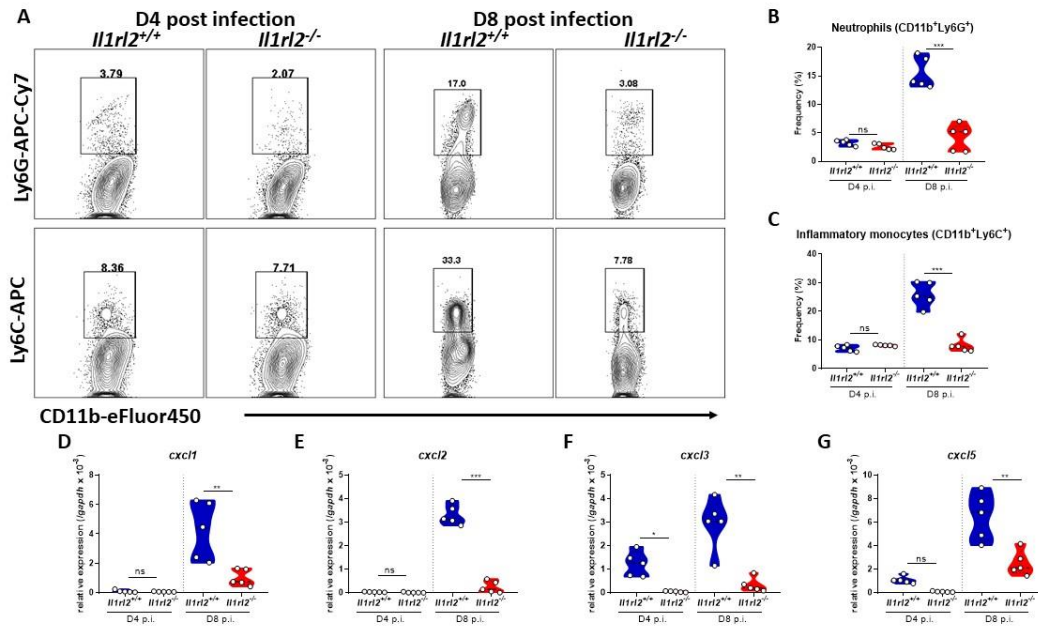


Figure 3.9 IL-36 signaling promotes innate immune cells infiltration during *C. rodentium* infection.

(A) Colonic lamina propria cells of infected *Il1rl2*^{+/+} and *Il1rl2*^{-/-} mice were isolated on the indicated days post infection and analyzed by flow cytometry for the infiltration of inflammatory neutrophil CD11b⁺Ly6G⁺ and monocytes CD11b⁺Ly6C⁺. (B and C) FACs frequency data for lamina propria cells of infected *Il1rl2*^{+/+} and *Il1rl2*^{-/-} mice generated as in (A). (D to G) Expression level neutrophil and monocytes chemoattractant (D) *cxcl1* (E) *cxcl2* (F) *cxcl3* and (G) *cxcl5* in large intestine from infected *Il1rl2*^{+/+} and *Il1rl2*^{-/-} mice. Data are representative of two independent experiments with 5 mice per group. All data presented as mean \pm SEM (one-way ANOVA with Tukey's multiple comparison test. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; ns, not significant).

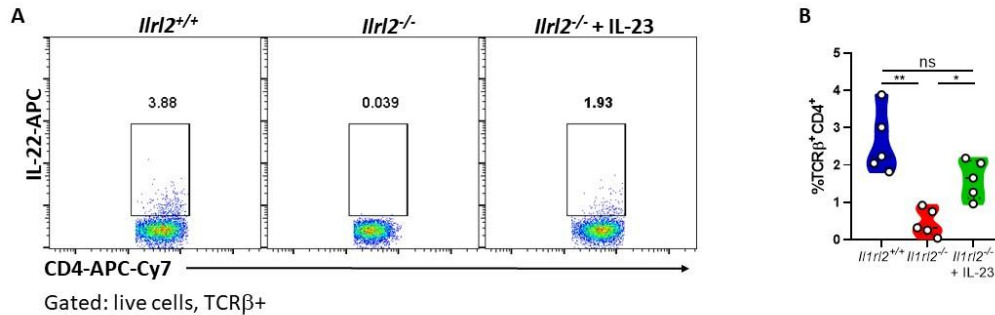


Figure 3.10 Early administration of exogenous IL-23 to *Il1rl2*^{-/-} mice infected *C. rodentium* induces modest IL-22 production from CD4⁺ T cells.

(A) Colonic lamina propria cells of *C. rodentium* infected *Il1rl2*^{+/+} and *Il1rl2*^{-/-} mice, in the presence/absence of IL-23 were isolated on day four post infection and analyzed by FACS for expression of intracellular IL-22 by TCRβ⁺CD4⁺ T gated cells. **(B)** FACS frequency data from LP cells of *C. rodentium* infected mice as shown in (A). Data are representative of three independent experiments with 5 mice per group. All data presented as mean ± SEM. (one-way ANOVA with Tukey's multiple comparison test. **P* < 0.5; ***P* < 0.05; ns, not significant).

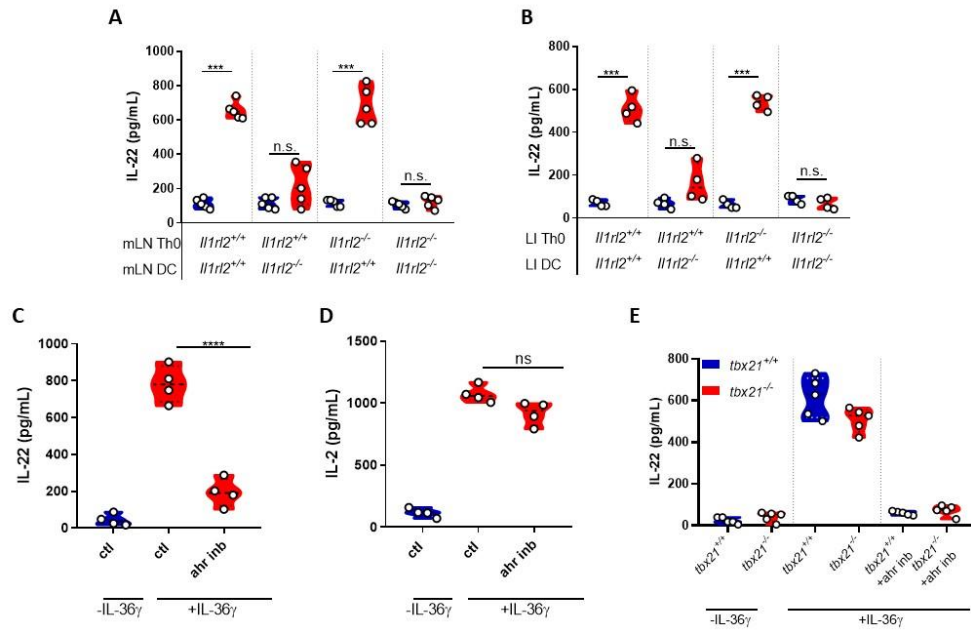


Figure 3.11 IL-36 signaling drives IL-22 induction in CD4⁺ T cells by stimulating AHR.

(A and B) FACS sorted naïve CD4⁺ T cells and DCs were co-cultured for 80 hours in the presence of IL-36γ and AHR inhibitor, CH-223191. **(A)** IL-22 protein, **(B)** IL-2 protein (B) in supernatants were assessed by ELISA. **(C)** FACS sorted naïve CD4⁺ T cells and DCs from *tbx21*^{+/+} and *tbx21*^{-/-} were co-cultured and stimulated with IL-36γ for 80 hours; in some-cultured AHR inhibitor, CH-223191 were added. IL-22 protein expression in supernatant were assessed. Data are representative of three independent experiments with 4-5 replicates. All data presented as mean ± SEM (one-way ANOVA with Tukey's multiple comparison test. **P* < 0.05, ***P* < 0.01, ****P* < 0.001; ns, not significant).

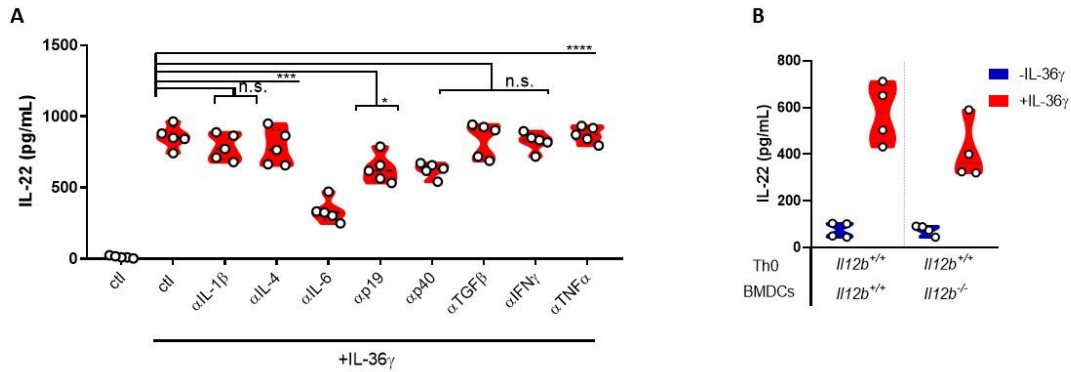


Figure 3.12 IL-6 induces IL-22 production in CD4⁺ T cells via IL-6

(A) FACS-sorted naïve CD4⁺ T cells and DCs were co-cultured for 80 hours in the presence of IL-36 γ and supplemented by indicated neutralizing antibodies for specific cytokines. IL-22 protein in supernatant was determined by ELISA. **(B)** FACS-sorted naïve CD4⁺ T cells and BMDCs were co-cultured using indicated cells from *Il12b*^{+/+} and *Il12b*^{-/-} mice with presence of IL-36 γ . IL-22 protein in the supernatant was assessed by ELISA. Data are representative of three independent experiments with 4-5 replicates. All data presented as mean \pm SEM (one-way ANOVA with Tukey's multiple comparison test. * P < 0.05, ** P < 0.01, *** P < 0.001; ns, not significant).

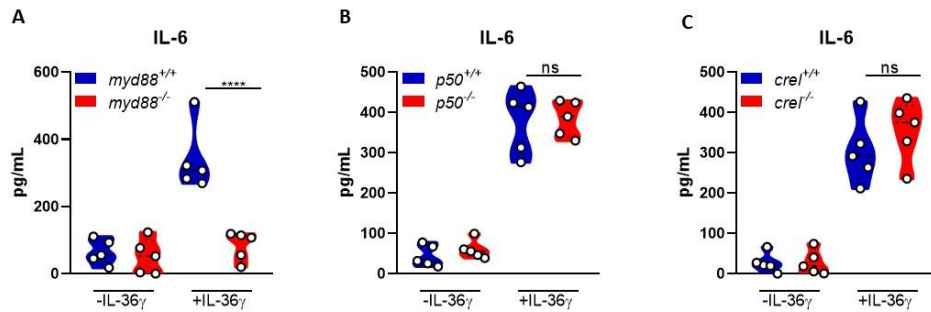


Figure 3.13 IL-36 signaling via MyD88 and independent of NF κ B p50 and NF κ B c-rel subunit

(A to C) BMDCs generated from wild-type, (A) *myd88*^{-/-}, (B) *p50*^{-/-} (B) or (C) *c-rel*^{-/-} were stimulated with IL-36 γ for 24 hours. IL-6 in supernatant were determined by ELISA. Data are representative of three independent experiments with 4-5 replicates. All data presented as mean \pm SEM (one-way ANOVA with Tukey's multiple comparison test. **** $P < 0.0001$; ns, not significant).

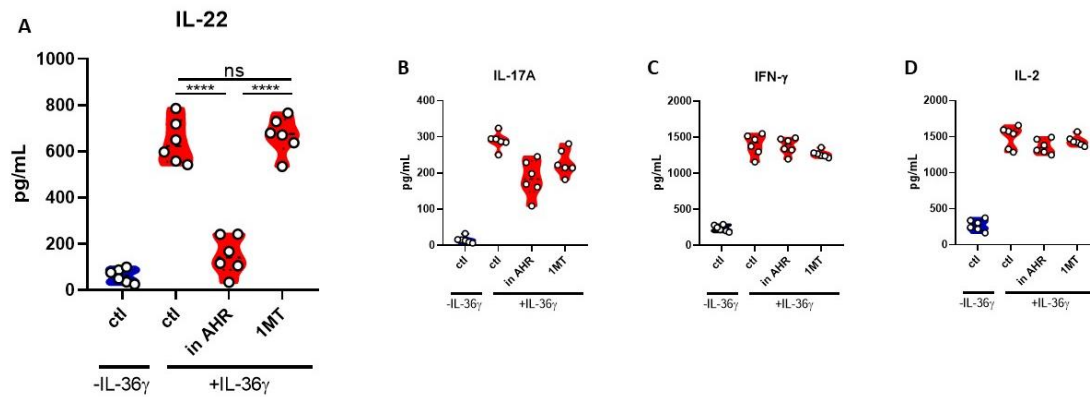


Figure 3.14 IL-36 induces IL-22 secretion by T cells is independent of kynurenine pathway

(A to D) FACS-sorted naïve CD4⁺ T cells and DCs were co-cultured and stimulated with IL-36 γ for 80 hours. In some cultured, AHR inhibitor or 1-MT were added. **(A)** IL-22 **(A)** IL-17A, **(C)** IFN- γ , and **(D)** IL-2 were assessed by ELISA. Data are representative of three independent experiments with 4-5 replicates. All data presented as mean \pm SEM (one-way ANOVA with Tukey's multiple comparison test. **** $P < 0.0001$; ns, not significant).

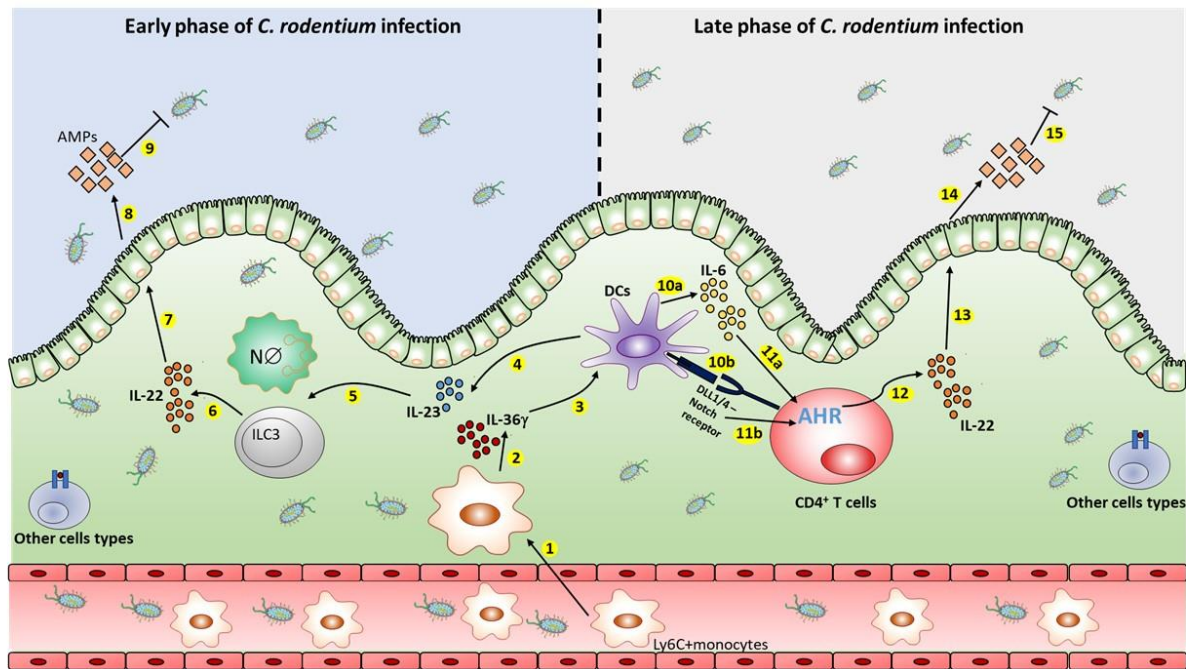


Figure 3.15 Proposed model of IL-36R/IL-22 axis for host protection against intestinal bacterial infection.

C. rodentium infection stimulated inflammatory Ly6C⁺ monocytes migrated from bloodstream to the colon (1) to produce IL-36γ (2). IL-36γ binds to its receptor complex on DCs (3) and induces the production of IL-23 (4). In the early phase of infection, IL-23 binds to its receptor on ILC3/neutrophil (5) and stimulates the secretion of IL-22 (6). IL-22 acts on IECs (7) to induce anti-microbial peptide (8) to ward off bacteria from entering the intestine (9). In the late phase of infection, IL-36γ stimulates DCs to produce IL-6 (10a) or notch ligands DLL1/4 (10b). IL-6 and DLL1/4 bind to their respective receptors on CD4⁺ T cells (11a, 11b) and activate AHR. AHR activation leads to the differentiation of naïve CD4⁺ T cells into IL-22-producing T cells (12). IL-22 from T cells acts on IECs (13) to induce AMPs (14, 15).

4 CHAPTER 4: INACTIVATION OF IL-36R SIGNALING AMILORATES CHRONIC INTESTINAL INFLAMMATION INDUCES BY *HELICOBACTER HEPATICUS*

4.1 Introduction

Inflammatory bowel disease (IBD), comprising ulcerative colitis and Crohn's disease, is a chronic inflammatory disorder of the gastrointestinal inflammation caused by abnormal immune responses to gut flora in the genetically susceptible host. Increased attachment of gut bacteria to the intestinal epithelium has been documented in IBD. The hallmark of active IBD is an abnormal mucosal infiltration by innate and adaptive immune cells and adaptive immune cells. Effector CD4⁺ T cells are critical in the defense against pathogens, whereas regulatory T are a critical in limiting the overactivity of CD4⁺ effector T cells [24]. IBD occurs when the balance between effector T cells and regulatory T cells is disturbed. Over the last few decades, the T_H1 and T_H17 immune reaction and secretion of pro-inflammatory cytokines are implicated in the pathogenesis of IBD, especially CD.

The relatively "newest" member of interleukin (IL)-1 cytokine superfamily, IL-36, has a crucial role in maintaining intestinal homeostasis. IL-36 agonists have been showed to be elevated in the inflamed gastrointestinal tissues in both human and animal experimental IBD models [43, 45, 46, 55, 56]. In DSS induces chronic intestinal inflammation, IL-36 α derived from CD163⁺, CD14⁺, and CD64⁺ macrophages regulate the production of pro-inflammatory cytokines and collagen type VI results in tissue fibrosis. Consequently, defective IL-36R signaling or preventive blockade of IL-36R signaling decreased intestinal inflammation and prevent fibrosis [2, 46].

However, little information is currently available on the relative contribution of IL-36R signaling on T cell dependent chronic intestinal inflammation. Here, we used the model of *Helicobacter hepaticus* induced T cells dependent on chronic intestinal inflammation. We

observed that mice with inactivation of IL-36R signaling are protected from effector T cell driven chronic intestinal inflammation and displayed higher colonic regulatory T cell (T_{reg}) with reduced T_H1 and T_H17 cells.

4.2 Materials and Methods

4.2.1 Mice

Wild-type C57BL/6 (B6 WT) were obtained from Jackson Laboratories. IL-36R^{-/-} mice (*Il1rl2*^{-/-}) on the C57BL/6 background (backcrossed >9 generations) were originally provided by Amgen.

4.2.2 *H. hepaticus* induces chronic colitis

Hh NCI-Frederick isolate 1A (strain 51449) was grown on blood agar plates (Thermo Scientific) under microaerophilic conditions. Mice were fed 4×10^9 colony forming units (CFU) of *H. hepaticus* by oral gavage at day 0, 2 and 4. In addition, 0.5mg of an IL-10R blocking antibody clone 1B1.3A was administered as an intraperitoneal injection twice weekly starting at day 0 of *Hh* infection.

4.2.3 Real-time PCR

Total RNA was isolated from mice colonic tissues using the Qiagen RNeasy Mini Kit according to manufacturer's protocol. cDNA was generated with Superscript First Strand Synthesis kit according to manufacturer's protocol. Q-PCR was performed with SYBR green (BioRad, CA) using a StepOnePlus PCR system (Applied Biosystem, CA) and *gapdh* were used to normalizes gene expression.

4.2.4 Flow cytometry

Fluorescence dye labeled antibodies (Abs) specific for TCRb, CD4, IFN-g, IL-17, FoxP3 were purchased from Becton Dickinson (BD), Biolegend, eBioscience. Fc block (2.4G2) was

purchased from BioXcel. Dead cells were stained using the fixable Aqua dead cells staining kit (Invitrogen). Intracellular staining done after stimulation of cells with phorbol-12-myristate 13-acetate and ionomycin (Sigma), and brefeldin A (eBioscience) for five hours. Stimulated cells were fix and permeabilized overnight and stained with antibodies specific for IFN- γ , IL-17 and FoxP3.

4.2.5 Histology

Colon tissues were fixed in 10% neutral formalin buffer. Paraffin embedding, sectioning and hematoxylin/eosin staining, and slide scanning were performed at Histowiz, Inc. (New York)

4.2.6 Statically Analysis

All statistical analyses were performed with GraphPad Prism software, version 8.0 (Graphpad Software). ONE-way ANOVA and Tukey's Multiple Comparison Test or Student's *t* test were used to determine significance. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$; n.s.= not significant.

4.3 Results and Discussion

4.3.1 IL-36 cytokines are expressed during *H. hepaticus* induces intestinal inflammation.

To determine the expression level of IL-36 agonists following *H. hepaticus* induces chronic intestinal inflammation, wild-type mice were infected with *H. hepaticus* strain ATCC 51449 and the expression of Il1f6 (IL-36 α), IL-1f8 (IL-36 β), and IL-1f9 (IL-36 γ) were assessed (Figure 4.1A). Both IL-36 α and IL-36 γ mRNA levels were significantly induced in *H. hepaticus* infected large intestine at five weeks post-infection. No IL-36 β mRNA was detected in *H. hepaticus* infected large intestine. (Figure 4.1 B)

4.3.2 Inactivation of IL-36R signaling *in vivo* ameliorates T cell driven intestinal inflammation.

Previous studies have shown IL-36R signaling induced T_H1 and T_H17 cell differentiation *in vitro* [66, 67]; thus, we explored the role of the IL-36R signaling in T cell-dependent intestinal inflammation induced by *H. hepaticus*. *H. hepaticus* induced intestinal inflammation have both been described as Th1-like because inflammatory cells produce IFN- γ . We used mice deficient of IL-36R (*Il1rl2*^{-/-}) to assess the contribution of IL-36R signaling in driving intestinal inflammation in this model. In responses to *H. hepaticus* infection, *Il1rl2*^{-/-} mice experienced normal body weight gains and have less lipocalin-2 (lcn-2) which is a marker for inflammation in intestinal inflammation, and less colitis score (Figure 4.2A-C).

Next, we determined the contribution of IL-36R signaling mediated regulation of effector T cell and regulatory T cell *in vivo* during intestinal inflammation. Following the infection of *H. hepaticus*, *Il1rl2*^{-/-} mice exhibited significantly reduction in IFN- γ , IL-17A production by colonic T cells when compared that of mice. Moreover, the frequency of CD4⁺FoxP3⁺ regulatory T cell was significantly elevated in *Il1rl2*^{-/-} mice (Figure 4.3 A and B). In addition, altered in IL-36R signaling pathway also resulted in decreased expression of proinflammatory genes including IL-12, IL-23, IL-6 in both colon and caecum infected mice (Figure 4.4 A and B).

T cells are one of the key initiators in the IBD. CD4⁺ T cells are enriched in the tissue from IBD patients with CD and UC, and blockade T cell trafficking molecules or depletion of CD4⁺ T cell is, in some cases, helpful to treating patients with IBD. In these studies, CD4⁺ T cell-depleting and blocking antibodies caused remission from the disease in several CD, and UC patients examined, suggesting a role of CD4⁺ T cells in propagating disease [2, 7, 24]. Numerous studies link chronic intestinal inflammation in IBD patients with increased levels of IL-36 cytokines [45,

46]. However, there is a lack of preclinical research into the role of IL-36 cytokines in promoting T cells dependent chronic intestinal inflammation. Here, using a bacterial driven T cell dependent intestinal inflammation, we dissected the role of IL-36R in chronic intestinal inflammation. The chronic intestinal inflammation develops in *H. hepaticus*-infected mice is associated with the elevation of IL-12, IL-23, and T_H1 response to the bacterium. In this model, mice lack IL-36R signaling exhibited less proinflammatory cytokines expression, increased T_{reg}, and diminished T_H1, and T_H17 CD4⁺ T cells resulted in significantly ameliorated intestinal inflammation. These data highlight the contribution of IL-36R in T cell driven chronic intestinal inflammation in mice and marked IL-36/IL-36R axis as an attractive therapeutic target to treat IBD.

4.4 Figures and Figures Legends

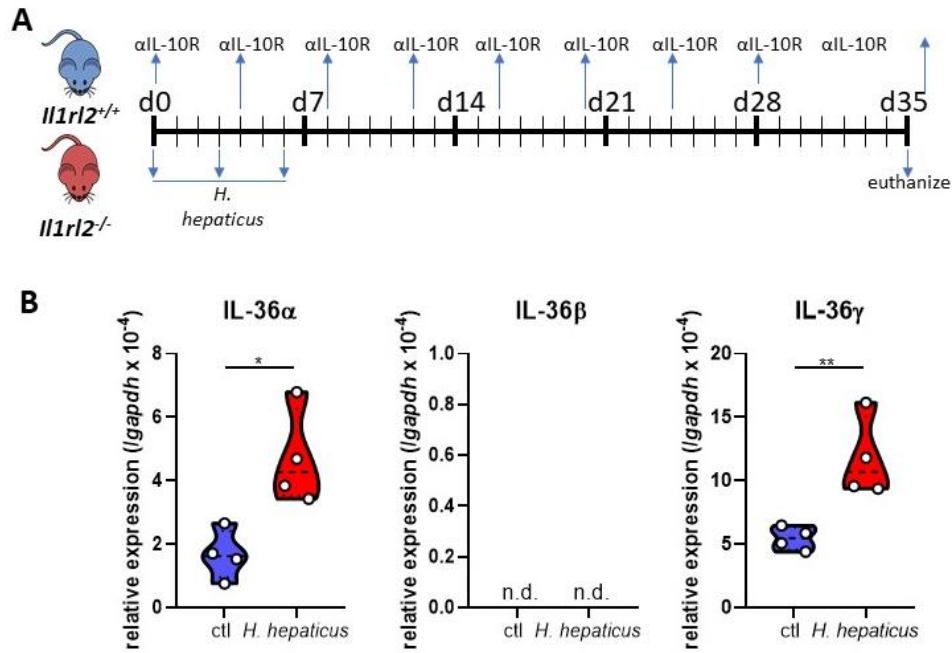


Figure 4.1 IL-36 α and IL-36 γ mRNA are expressed during *H. hepaticus* induces intestinal inflammation.

(A) Experimental schematic of intestinal bacterial infection by gastric gavage mice with *H. hepaticus*. (B) IL-36 α , IL-36 β , and IL-36 γ mRNA expression in colonic tissues of wild-type mice infected *H. hepaticus* at 35 days post infection.

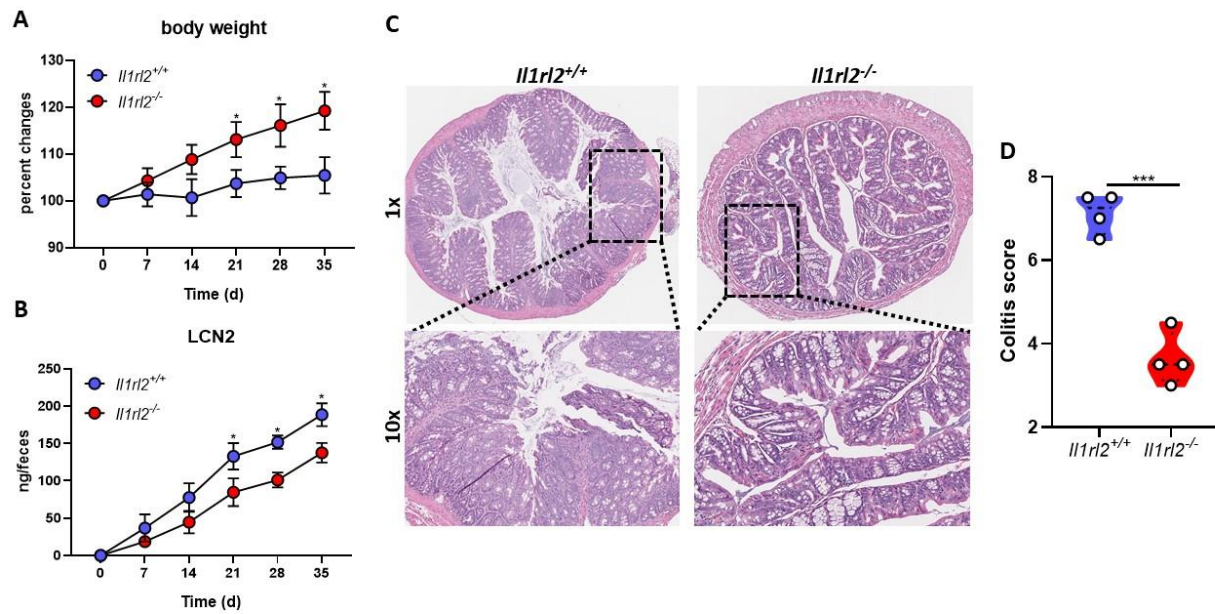


Figure 4.2 Inactivation of IL-36R signaling ameliorates chronic intestinal inflammation induced by *H. hepaticus*.

Four to six-week-old *Il1rl2*^{+/+} and *Il1rl2*^{-/-} mice infected with *H. hepaticus* by gastric gavage. (A) average body weight change. (B) average lcn-2 in mice feces at indicated time point. (C and D) The H&E staining and histology scoring of colon section from infected mice at 35 days post infection

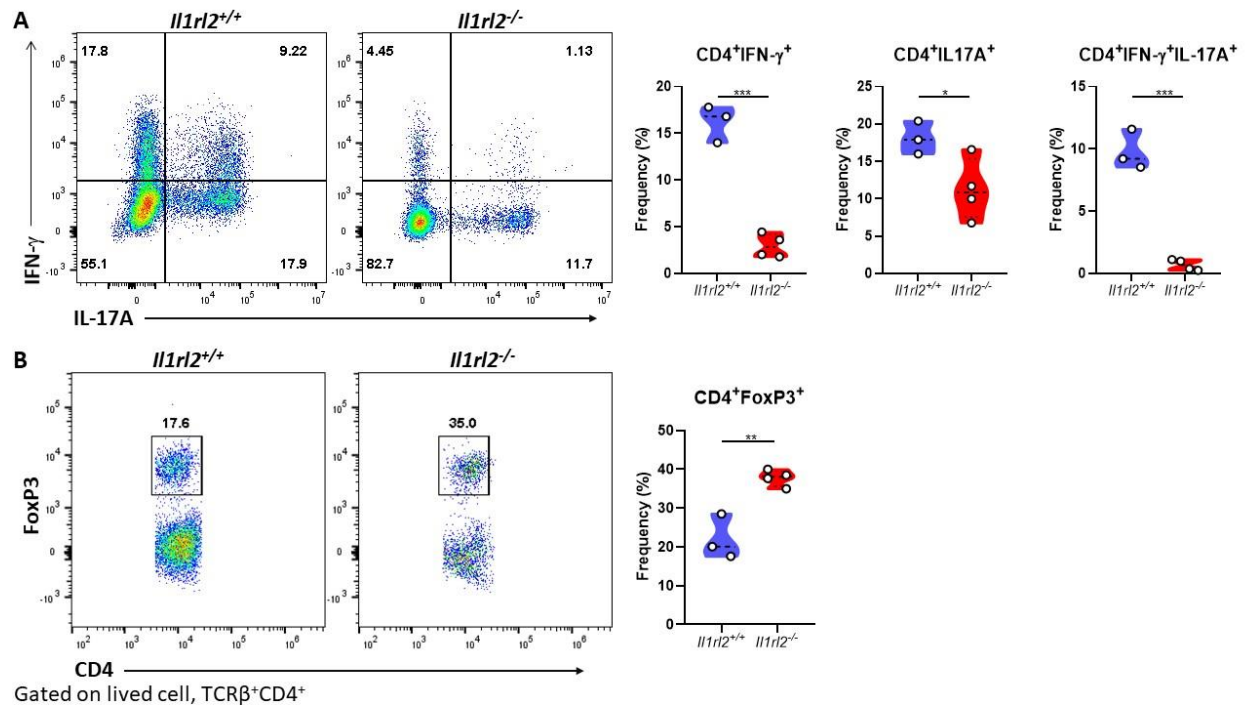


Figure 4.3 Inactivation of IL-36R signaling enhance T_H1/T_H17 and inhibit FoxP3 differentiation.

Four to six-week-old *Il1rl2*^{+/+} and *Il1rl2*^{-/-} mice infected with *H. hepaticus* by gastric gavage. **(A and B)** FACS plot and FACS frequency data of colonic lamina propria cells of *H. hepaticus* infected mice at 35 days post infection were isolated analyzed by FACS expression of intracellular IFN- γ , IL-17 and FoxP3 by TCR β ⁺CD4⁺ gated cells.

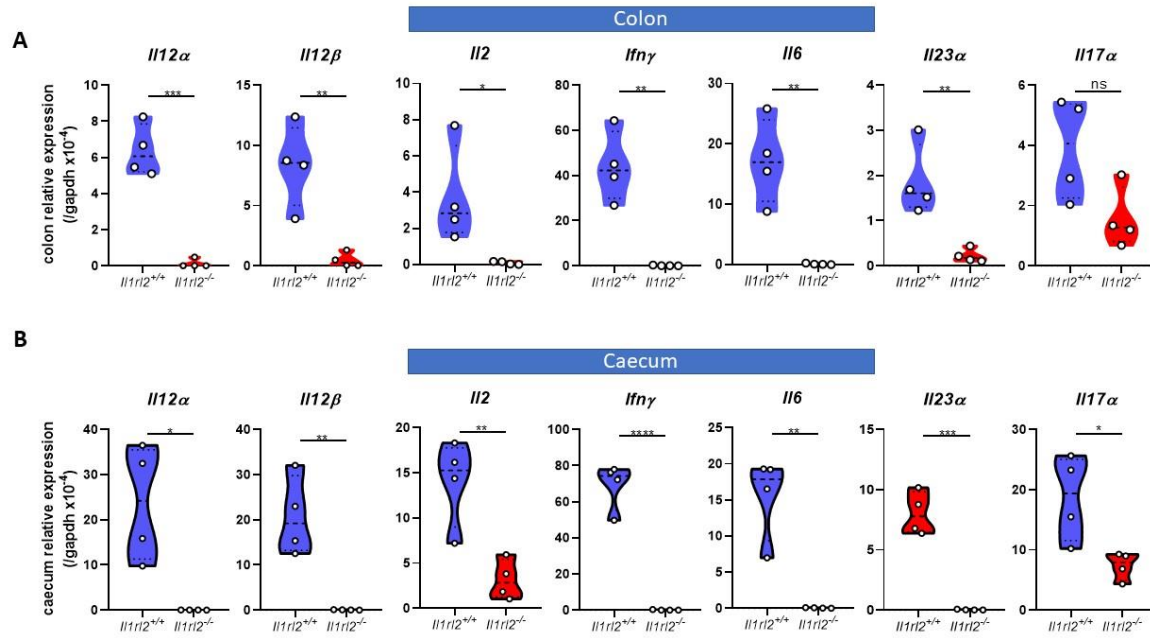


Figure 4.4 IL-36R signaling modulates the production of cytokines in mice infected with *H. hepaticus*.

mRNA expression of proinflammatory cytokines from (A) colon (B) caecum of mice infected with *H. hepaticus*.

5 CHAPTER 5: CONCLUSION AND FUTURE STUDIES

IL-36 cytokines are members of the IL-1 superfamily, although these cytokines were discovered almost twenty years ago, their functions remain enigmatic within the field of IBD. Recent work from our lab, and others, has unraveled the significant and complex contributions of the IL-36R signaling in mucosal immune responses during intestinal inflammation. We reported that IL-36 cytokines are expressed in multiple models of murine intestinal inflammation and during human IBD. We have shown that IL-36 ligands, especially IL-36 γ , are secreted by Ly6C⁺ inflammatory monocytes in response to bacterial translocation caused by intestinal barrier damage, in mice. Like other members in IL-1 superfamily, IL-36 cytokines have a dichotomous role in intestinal inflammation (Figure 5.1). In the context of acute intestinal inflammation, several groups and we have observed *Il1rl2*^{-/-} mice have reduced disease severity in the damage phase and correlated with a decrease of innate inflammatory cells such as neutrophil, monocytes, and macrophages.

Interestingly, in the healing phase of acute intestinal injury, *Il1rl2*^{-/-} mice exhibited defective recovery and impaired wound healing from DSS-induced damage. IL-36 mediated mucosal healing occurs via two mechanisms: (1) IL-36 induces the expression of IL-23/IL-22 and antimicrobial peptides. (2) IL-36 induces the proliferation of intestinal epithelial cells and activates fibroblasts. Upon tissue injury in the colon, IL-36 α and IL-36 γ are released from inflammatory macrophage and intestinal epithelial cells. Subsequently, these IL-36 ligands bind to IL-36R complex on target cells, including DCs, colonic fibroblasts, and intestinal epithelial cells (IECs); thereby, initiating effector mechanism that stimulates resolution of intestinal damage. IL-36 binds to its receptors on DCs and signals through MyD88 protein adaptor which activates NF κ B, c-rel, and p50 subunits which induce the expression of IL-23. Following the secretion of IL-23,

numerous cell types in the colon express IL-23R and can produce of IL-22. IL-22 then stimulates the restitution of IEC and the generation of antimicrobial peptides. Another mechanism of mucosal healing that is dependent on IL-36R signaling is IL-36 ligand activation of IL-36R+ colonic fibroblasts which induce expression of granulocyte-macrophage-stimulating factor (GM-CSF) and IL-6. Both GM-CSF and IL-6 are essential in the restoration of epithelial integrity after mucosal damages. Moreover, in vivo treatment with IL-36R ligands or IL-23 significantly accelerated mucosal healing. We have also demonstrated the importance of IL-36R signaling in host protection against intestinal bacterial infection. In the context of bacterial-induced intestinal inflammation, IL-36R deficiency resulted in reduced host ability to control enteropathogenic bacteria *C. rodentium* infection. This defective phenotype of *Il1rl2*^{-/-} mice is associated with a decrease in recruitment of innate inflammatory cells and proinflammation cytokines, including IL-23, IL-6, and IL-22.

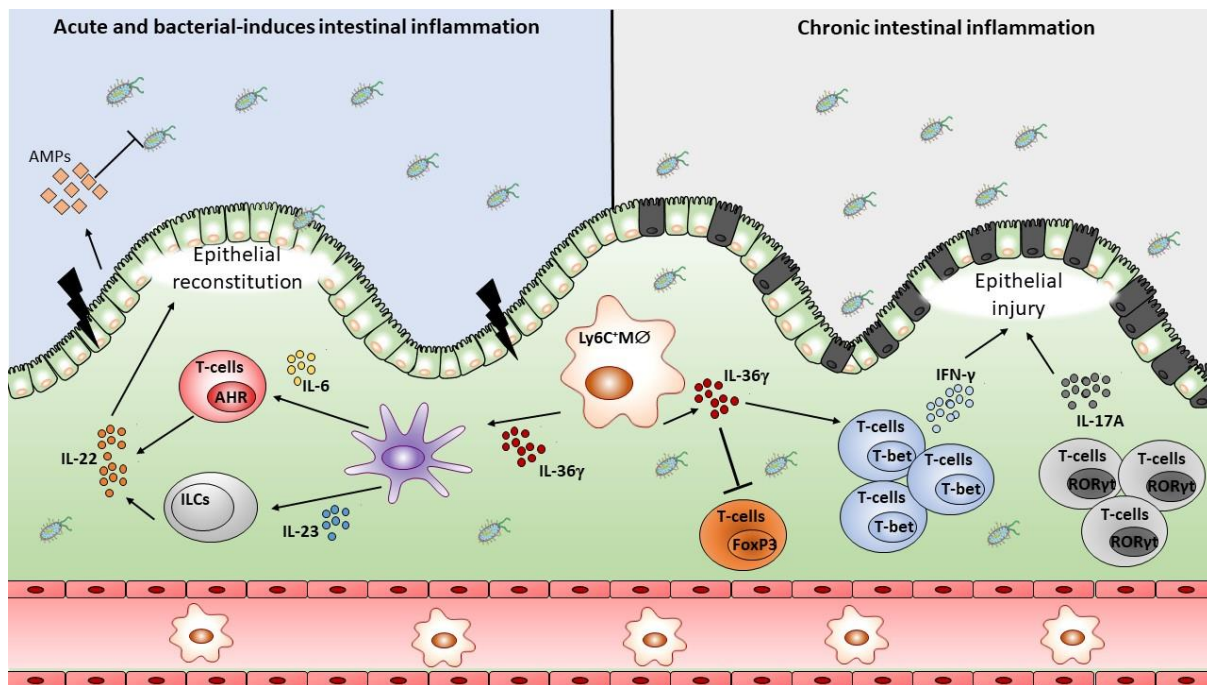


Figure 5.1 The dichotomous roles of IL-36R signaling in intestinal inflammation

In contrast to acute and bacterial-induced intestinal inflammation, IL-36R signaling is pathogenic in chronic intestinal inflammation. We reported in the oxazolone and *H. hepaticus* models of colitis, signaling through the IL-36R complex inhibits Foxp3⁺ regulatory T cell (T_{reg}) expansion and upregulates the differentiation of naïve CD4⁺ T cells into T_H1, T_H17, and T_H9 effector T cells. Mice with inactivated IL-36R are protected from effector T cell driven intestinal inflammation and display higher colonic T_{reg} cells and reduced T_H1/T_H17/T_H9 cells. We also investigated the impact of IL-36R signaling in the T cell transfer model of chronic intestinal inflammation. Naïve T cells (CD4⁺CD45RB^{high}) were isolated from *Il1rl2*^{+/+} and *Il1rl2*^{-/-} mice and transferred to recipients that lack T and B cells. In this model, *rag1*^{-/-} mice that received IL-36R-deficient CD45RB^{high} cells exhibited moderate weight loss and colonic inflammation when compared to *rag1*^{-/-} mice received IL-36R-deficient CD45RB^{hi} cells. Recently published by Scheibe *et al.*, revealed that tissue isolated from patients with IBD express a higher level of IL-36 α and collagens than healthy patients. In additional chronic intestinal inflammation models, including DSS and TNBS, IL-36 α derived from CD163⁺, CD14⁺, and CD64⁺ macrophages govern the production of proinflammatory cytokines and collagen type VI resulting in tissue fibrosis. Administration of IL-36R agonist ligands led to an increase in the number of α -smooth muscle actin-positive cells. Treatment of mice with antibody-mediated inhibition of IL-36R reversed the established intestinal fibrosis and ameliorated inflammation. Currently, clinical trials targeting IL-36R signaling with neutralizing antibodies are ongoing with IBD's patients.

Despite the use of inhibitory agents that target proinflammatory cytokines and immune cell trafficking molecules, many patients with IBD do not respond to therapy or show loss of clinical response over time. The experiments in the dissertation in combination with the results from other groups, has established that the IL-36/IL-36R axis has a role in inducing the protective function as

well as a role in pathogenic effector T_H cell differentiation. Therefore, IL-36 may be an attractive target during active chronic intestinal inflammation. However, it should be noted that this may lead to potential side effects, such as the increased risk for infections and impairment in barrier repair. It would be interesting to further research if simultaneously blocking IL-36R signaling and delivering pro-healing factors, such as IL-22 or pro-antimicrobial factors, could circumvent the complications that might arise when the IL-36/IL-36R axis is blocked.

REFERENCES

1. Guan, Q., *A Comprehensive Review and Update on the Pathogenesis of Inflammatory Bowel Disease*. J Immunol Res, 2019. **2019**: p. 7247238.
2. Neurath, M.F., *Targeting immune cell circuits and trafficking in inflammatory bowel disease*. Nat Immunol, 2019. **20**(8): p. 970-979.
3. Seyedian, S.S., F. Nokhostin, and M.D. Malimir, *A review of the diagnosis, prevention, and treatment methods of inflammatory bowel disease*. J Med Life, 2019. **12**(2): p. 113-122.
4. Alam, M.S., et al., *Notch signaling drives IL-22 secretion in CD4+ T cells by stimulating the aryl hydrocarbon receptor*. Proc Natl Acad Sci U S A, 2010. **107**(13): p. 5943-8.
5. Zelante, T., et al., *Tryptophan catabolites from microbiota engage aryl hydrocarbon receptor and balance mucosal reactivity via interleukin-22*. Immunity, 2013. **39**(2): p. 372-85.
6. M'Koma, A.E., *Inflammatory bowel disease: an expanding global health problem*. Clin Med Insights Gastroenterol, 2013. **6**: p. 33-47.
7. Neurath, M., *Current and emerging therapeutic targets for IBD*. Nat Rev Gastroenterol Hepatol, 2017. **14**(11): p. 688.
8. Neurath, M.F., *Cytokines in inflammatory bowel disease*. Nat Rev Immunol, 2014. **14**(5): p. 329-42.
9. Conley, S., et al., *Symptom clusters in adults with inflammatory bowel disease*. Res Nurs Health, 2017. **40**(5): p. 424-434.
10. Cao, Q., et al., *The prevalence and risk factors of psychological disorders, malnutrition and quality of life in IBD patients*. Scand J Gastroenterol, 2019. **54**(12): p. 1458-1466.
11. de Souza, H.S. and C. Fiocchi, *Immunopathogenesis of IBD: current state of the art*. Nat Rev Gastroenterol Hepatol, 2016. **13**(1): p. 13-27.
12. Kim, D.H. and J.H. Cheon, *Pathogenesis of Inflammatory Bowel Disease and Recent Advances in Biologic Therapies*. Immune Netw, 2017. **17**(1): p. 25-40.
13. Ko, Y., *Inflammatory bowel disease environmental risk factors versus genetics based on migration epidemiological studies*. J Gastroenterol Hepatol, 2018. **33 Suppl 3**: p. 22.
14. Leong, R.W., *Environmental risk factors in inflammatory bowel disease*. J Gastroenterol Hepatol, 2010. **25**(2): p. 227-8.
15. Molodecky, N.A. and G.G. Kaplan, *Environmental risk factors for inflammatory bowel disease*. Gastroenterol Hepatol (N Y), 2010. **6**(5): p. 339-46.
16. Halfvarson, J., et al., *Dynamics of the human gut microbiome in inflammatory bowel disease*. Nat Microbiol, 2017. **2**: p. 17004.
17. Neurath, M.F. and S. Finotto, *The many roads to inflammatory bowel diseases*. Immunity, 2006. **25**(2): p. 189-91.
18. Schreiner, P., et al., *Mechanism-Based Treatment Strategies for IBD: Cytokines, Cell Adhesion Molecules, JAK Inhibitors, Gut Flora, and More*. Inflamm Intest Dis, 2019. **4**(3): p. 79-96.
19. Wu, H.J. and E. Wu, *The role of gut microbiota in immune homeostasis and autoimmunity*. Gut Microbes, 2012. **3**(1): p. 4-14.
20. Zuo, T. and S.C. Ng, *The Gut Microbiota in the Pathogenesis and Therapeutics of Inflammatory Bowel Disease*. Front Microbiol, 2018. **9**: p. 2247.

21. Moschen, A.R., H. Tilg, and T. Raine, *IL-12, IL-23 and IL-17 in IBD: immunobiology and therapeutic targeting*. Nat Rev Gastroenterol Hepatol, 2019. **16**(3): p. 185-196.
22. Boden, E.K. and J.D. Lord, *CD4 T Cells in IBD: Crossing the Line?* Dig Dis Sci, 2017. **62**(9): p. 2208-2210.
23. Friedrich, M., M. Pohin, and F. Powrie, *Cytokine Networks in the Pathophysiology of Inflammatory Bowel Disease*. Immunity, 2019. **50**(4): p. 992-1006.
24. Imam, T., et al., *Effector T Helper Cell Subsets in Inflammatory Bowel Diseases*. Front Immunol, 2018. **9**: p. 1212.
25. Smids, C., et al., *Intestinal T Cell Profiling in Inflammatory Bowel Disease: Linking T Cell Subsets to Disease Activity and Disease Course*. J Crohns Colitis, 2018. **12**(4): p. 465-475.
26. Bevivino, G. and G. Monteleone, *Advances in understanding the role of cytokines in inflammatory bowel disease*. Expert Rev Gastroenterol Hepatol, 2018. **12**(9): p. 907-915.
27. Papadakis, K.A. and S.R. Targan, *Role of cytokines in the pathogenesis of inflammatory bowel disease*. Annu Rev Med, 2000. **51**: p. 289-98.
28. Sanchez-Munoz, F., A. Dominguez-Lopez, and J.K. Yamamoto-Furusho, *Role of cytokines in inflammatory bowel disease*. World J Gastroenterol, 2008. **14**(27): p. 4280-8.
29. Strober, W. and I.J. Fuss, *Proinflammatory cytokines in the pathogenesis of inflammatory bowel diseases*. Gastroenterology, 2011. **140**(6): p. 1756-1767.
30. Fields, J.K., S. Gunther, and E.J. Sundberg, *Structural Basis of IL-1 Family Cytokine Signaling*. Front Immunol, 2019. **10**: p. 1412.
31. Lovenberg, T.W., et al., *Cloning of a cDNA encoding a novel interleukin-1 receptor related protein (IL 1R-rp2)*. J Neuroimmunol, 1996. **70**(2): p. 113-22.
32. Buhl, A.L. and J. Wenzel, *Interleukin-36 in Infectious and Inflammatory Skin Diseases*. Front Immunol, 2019. **10**: p. 1162.
33. Dunn, E., et al., *Annotating genes with potential roles in the immune system: six new members of the IL-1 family*. Trends Immunol, 2001. **22**(10): p. 533-6.
34. Bassoy, E.Y., J.E. Towne, and C. Gabay, *Regulation and function of interleukin-36 cytokines*. Immunol Rev, 2018. **281**(1): p. 169-178.
35. Yuan, Z.C., et al., *Biology of IL-36 Signaling and Its Role in Systemic Inflammatory Diseases*. Front Immunol, 2019. **10**: p. 2532.
36. Boutet, M.A., A. Nerviani, and C. Pitzalis, *IL-36, IL-37, and IL-38 Cytokines in Skin and Joint Inflammation: A Comprehensive Review of Their Therapeutic Potential*. Int J Mol Sci, 2019. **20**(6).
37. Boutet, M.A., et al., *Interleukin-36 family dysregulation drives joint inflammation and therapy response in psoriatic arthritis*. Rheumatology (Oxford), 2019.
38. Catapano, M., et al., *IL-36 Promotes Systemic IFN- γ Responses in Severe Forms of Psoriasis*. J Invest Dermatol, 2019.
39. Mai, S.Z., et al., *Increased serum IL-36 α and IL-36 γ levels in patients with systemic lupus erythematosus: Association with disease activity and arthritis*. Int Immunopharmacol, 2018. **58**: p. 103-108.
40. Murrieta-Coxca, J.M., et al., *IL-36 Cytokines: Regulators of Inflammatory Responses and Their Emerging Role in Immunology of Reproduction*. Int J Mol Sci, 2019. **20**(7).
41. Ohko, K., et al., *IL-36 Signaling Is Essential for Psoriatic Inflammation through the Augmentation of Innate Immune Responses*. J Invest Dermatol, 2019. **139**(6): p. 1400-1404.

42. Queen, D., C. Ediriweera, and L. Liu, *Function and Regulation of IL-36 Signaling in Inflammatory Diseases and Cancer Development*. Front Cell Dev Biol, 2019. **7**: p. 317.
43. Russell, S.E., et al., *IL-36 α expression is elevated in ulcerative colitis and promotes colonic inflammation*. Mucosal Immunol, 2016. **9**(5): p. 1193-204.
44. Sato, S., et al., *Upregulation of IL-36 cytokines in folliculitis and eosinophilic pustular folliculitis*. Australas J Dermatol, 2019.
45. Scheibe, K., et al., *IL-36R signalling activates intestinal epithelial cells and fibroblasts and promotes mucosal healing in vivo*. Gut, 2017. **66**(5): p. 823-838.
46. Scheibe, K., et al., *Inhibiting Interleukin 36 Receptor Signaling Reduces Fibrosis in Mice With Chronic Intestinal Inflammation*. Gastroenterology, 2019. **156**(4): p. 1082-1097 e11.
47. Walsh, P.T. and P.G. Fallon, *The emergence of the IL-36 cytokine family as novel targets for inflammatory diseases*. Ann N Y Acad Sci, 2018. **1417**(1): p. 23-34.
48. Ding, L., et al., *IL-36 cytokines in autoimmunity and inflammatory disease*. Oncotarget, 2018. **9**(2): p. 2895-2901.
49. Gabay, C. and J.E. Towne, *Regulation and function of interleukin-36 cytokines in homeostasis and pathological conditions*. J Leukoc Biol, 2015. **97**(4): p. 645-52.
50. Aoyagi, T., et al., *IL-36 receptor deletion attenuates lung injury and decreases mortality in murine influenza pneumonia*. Mucosal Immunol, 2017. **10**(4): p. 1043-1055.
51. Aoyagi, T., et al., *Interleukin-36 γ and IL-36 receptor signaling mediate impaired host immunity and lung injury in cytotoxic Pseudomonas aeruginosa pulmonary infection: Role of prostaglandin E2*. PLoS Pathog, 2017. **13**(11): p. e1006737.
52. Wang, P., A.M. Gamero, and L.E. Jensen, *IL-36 promotes anti-viral immunity by boosting sensitivity to IFN- α /beta in IRF1 dependent and independent manners*. Nat Commun, 2019. **10**(1): p. 4700.
53. Wein, A.N., et al., *IL-36 γ Protects against Severe Influenza Infection by Promoting Lung Alveolar Macrophage Survival and Limiting Viral Replication*. J Immunol, 2018. **201**(2): p. 573-582.
54. Harusato, A., et al., *IL-36 γ signaling controls the induced regulatory T cell-Th9 cell balance via NF κ B activation and STAT transcription factors*. Mucosal Immunol, 2017. **10**(6): p. 1455-1467.
55. Medina-Contreras, O., et al., *Cutting Edge: IL-36 Receptor Promotes Resolution of Intestinal Damage*. J Immunol, 2016. **196**(1): p. 34-8.
56. Ngo, V.L., et al., *A cytokine network involving IL-36 γ , IL-23, and IL-22 promotes antimicrobial defense and recovery from intestinal barrier damage*. Proc Natl Acad Sci U S A, 2018. **115**(22): p. E5076-E5085.
57. Foster, A.M., et al., *IL-36 promotes myeloid cell infiltration, activation, and inflammatory activity in skin*. J Immunol, 2014. **192**(12): p. 6053-61.
58. Kovach, M.A., et al., *IL-36 γ is secreted in microparticles and exosomes by lung macrophages in response to bacteria and bacterial components*. J Leukoc Biol, 2016. **100**(2): p. 413-21.
59. Ainscough, J.S., et al., *Cathepsin S is the major activator of the psoriasis-associated proinflammatory cytokine IL-36 γ* . Proc Natl Acad Sci U S A, 2017. **114**(13): p. E2748-E2757.
60. Clancy, D.M., et al., *Production of biologically active IL-36 family cytokines through insertion of N-terminal caspase cleavage motifs*. FEBS Open Bio, 2016. **6**(4): p. 338-48.

61. Lian, L.H., et al., *The double-stranded RNA analogue polyinosinic-polycytidylic acid induces keratinocyte pyroptosis and release of IL-36gamma*. J Invest Dermatol, 2012. **132**(5): p. 1346-53.
62. Muller, A., et al., *IkappaBzeta is a key transcriptional regulator of IL-36-driven psoriasis-related gene expression in keratinocytes*. Proc Natl Acad Sci U S A, 2018. **115**(40): p. 10088-10093.
63. Guo, J., et al., *Cathepsin G cleaves and activates IL-36gamma and promotes the inflammation of psoriasis*. Drug Des Devel Ther, 2019. **13**: p. 581-588.
64. Sullivan, G.P., et al., *Suppressing IL-36-driven inflammation using peptide pseudosubstrates for neutrophil proteases*. Cell Death Dis, 2018. **9**(3): p. 378.
65. van de Veerdonk, F.L., et al., *IL-38 binds to the IL-36 receptor and has biological effects on immune cells similar to IL-36 receptor antagonist*. Proc Natl Acad Sci U S A, 2012. **109**(8): p. 3001-5.
66. Vigne, S., et al., *IL-36R ligands are potent regulators of dendritic and T cells*. Blood, 2011. **118**(22): p. 5813-5823.
67. Vigne, S., et al., *IL-36 signaling amplifies Th1 responses by enhancing proliferation and Th1 polarization of naive CD4+T cells*. Cytokine, 2012. **59**(3): p. 577-578.
68. Sonnenberg, G.F., L.A. Fouser, and D. Artis, *Border patrol: regulation of immunity, inflammation and tissue homeostasis at barrier surfaces by IL-22*. Nat Immunol, 2011. **12**(5): p. 383-90.
69. Turner, J.R., *Intestinal mucosal barrier function in health and disease*. Nat Rev Immunol, 2009. **9**(11): p. 799-809.
70. Sonnenberg, G.F., L.A. Fouser, and D. Artis, *Functional biology of the IL-22-IL-22R pathway in regulating immunity and inflammation at barrier surfaces*. Adv Immunol, 2010. **107**: p. 1-29.
71. Dudakov, J.A., A.M. Hanash, and M.R. van den Brink, *Interleukin-22: immunobiology and pathology*. Annu Rev Immunol, 2015. **33**: p. 747-85.
72. Sugimoto, K., et al., *IL-22 ameliorates intestinal inflammation in a mouse model of ulcerative colitis*. J Clin Invest, 2008. **118**(2): p. 534-44.
73. Zenewicz, L.A., et al., *Innate and adaptive interleukin-22 protects mice from inflammatory bowel disease*. Immunity, 2008. **29**(6): p. 947-57.
74. Zheng, Y., et al., *Interleukin-22 mediates early host defense against attaching and effacing bacterial pathogens*. Nat Med, 2008. **14**(3): p. 282-9.
75. Zindl, C.L., et al., *IL-22-producing neutrophils contribute to antimicrobial defense and restitution of colonic epithelial integrity during colitis*. Proc Natl Acad Sci U S A, 2013. **110**(31): p. 12768-73.
76. Eidenschenk, C., et al., *Role of IL-22 in microbial host defense*. Curr Top Microbiol Immunol, 2014. **380**: p. 213-36.
77. Rutz, S., X. Wang, and W. Ouyang, *The IL-20 subfamily of cytokines--from host defence to tissue homeostasis*. Nat Rev Immunol, 2014. **14**(12): p. 783-95.
78. Kinnebrew, M.A., et al., *Interleukin 23 production by intestinal CD103(+)CD11b(+) dendritic cells in response to bacterial flagellin enhances mucosal innate immune defense*. Immunity, 2012. **36**(2): p. 276-87.
79. Shih, V.F., et al., *Homeostatic IL-23 receptor signaling limits Th17 response through IL-22-mediated containment of commensal microbiota*. Proc Natl Acad Sci U S A, 2014. **111**(38): p. 13942-7.

80. Garlanda, C., C.A. Dinarello, and A. Mantovani, *The interleukin-1 family: back to the future*. Immunity, 2013. **39**(6): p. 1003-18.
81. Lopetuso, L.R., S. Chowdhry, and T.T. Pizarro, *Opposing Functions of Classic and Novel IL-1 Family Members in Gut Health and Disease*. Front Immunol, 2013. **4**: p. 181.
82. Bamias, G., et al., *New insights into the dichotomous role of innate cytokines in gut homeostasis and inflammation*. Cytokine, 2012. **59**(3): p. 451-9.
83. Bamias, G. and F. Cominelli, *Cytokines and intestinal inflammation*. Curr Opin Gastroenterol, 2016. **32**(6): p. 437-442.
84. Walsh, P.T. and P.G. Fallon, *The emergence of the IL-36 cytokine family as novel targets for inflammatory diseases*. Ann N Y Acad Sci, 2016.
85. Scheibe, K., et al., *IL-36R signalling activates intestinal epithelial cells and fibroblasts and promotes mucosal healing in vivo*. Gut, 2016.
86. Nishida, A., et al., *Increased Expression of Interleukin-36, a Member of the Interleukin-1 Cytokine Family, in Inflammatory Bowel Disease*. Inflamm Bowel Dis, 2016. **22**(2): p. 303-14.
87. Boutet, M.A., et al., *Distinct expression of interleukin (IL)-36alpha, beta and gamma, their antagonist IL-36Ra and IL-38 in psoriasis, rheumatoid arthritis and Crohn's disease*. Clin Exp Immunol, 2016. **184**(2): p. 159-73.
88. Friedrich, M., et al., *IL-36gamma sustains a proinflammatory self-amplifying loop with IL-17C in anti-TNF-induced psoriasiform skin lesions of patients with Crohn's disease*. Inflamm Bowel Dis, 2014. **20**(11): p. 1891-901.
89. Takahashi, K., et al., *Interleukin (IL)-1beta Is a Strong Inducer of IL-36gamma Expression in Human Colonic Myofibroblasts*. PLoS One, 2015. **10**(11): p. e0138423.
90. Kanda, T., et al., *Interleukin(IL)-36alpha and IL-36gamma Induce Proinflammatory Mediators from Human Colonic Subepithelial Myofibroblasts*. Front Med (Lausanne), 2015. **2**: p. 69.
91. Satpathy, A.T., et al., *Notch2-dependent classical dendritic cells orchestrate intestinal immunity to attaching-and-effacing bacterial pathogens*. Nat Immunol, 2013. **14**(9): p. 937-48.
92. Jiang, Z., et al., *IL-36gamma Induced by the TLR3-SLUG-VDR Axis Promotes Wound Healing via REG3A*. J Invest Dermatol, 2017.
93. Denning, T.L., et al., *Functional specializations of intestinal dendritic cell and macrophage subsets that control Th17 and regulatory T cell responses are dependent on the T cell/APC ratio, source of mouse strain, and regional localization*. J Immunol, 2011. **187**(2): p. 733-47.
94. MacDonald, K.P., et al., *An antibody against the colony-stimulating factor 1 receptor depletes the resident subset of monocytes and tissue- and tumor-associated macrophages but does not inhibit inflammation*. Blood, 2010. **116**(19): p. 3955-63.
95. Carmody, R.J., et al., *Essential roles of c-Rel in TLR-induced IL-23 p19 gene expression in dendritic cells*. J Immunol, 2007. **178**(1): p. 186-91.
96. Peterson, L.W. and D. Artis, *Intestinal epithelial cells: regulators of barrier function and immune homeostasis*. Nat Rev Immunol, 2014. **14**(3): p. 141-53.
97. Luissint, A.C., C.A. Parkos, and A. Nusrat, *Inflammation and the Intestinal Barrier: Leukocyte-Epithelial Cell Interactions, Cell Junction Remodeling, and Mucosal Repair*. Gastroenterology, 2016. **151**(4): p. 616-32.

98. Round, J.L. and S.K. Mazmanian, *The gut microbiota shapes intestinal immune responses during health and disease*. Nat Rev Immunol, 2009. **9**(5): p. 313-23.
99. Zhou, L. and G.F. Sonnenberg, *Essential immunologic orchestrators of intestinal homeostasis*. Sci Immunol, 2018. **3**(20).
100. Hooper, L.V., D.R. Littman, and A.J. Macpherson, *Interactions between the microbiota and the immune system*. Science, 2012. **336**(6086): p. 1268-73.
101. Aden, K., et al., *Epithelial IL-23R Signaling Licenses Protective IL-22 Responses in Intestinal Inflammation*. Cell Rep, 2016. **16**(8): p. 2208-18.
102. Zenewicz, L.A., et al., *IL-22 deficiency alters colonic microbiota to be transmissible and colitogenic*. J Immunol, 2013. **190**(10): p. 5306-12.
103. Palomo, J., et al., *The interleukin (IL)-1 cytokine family--Balance between agonists and antagonists in inflammatory diseases*. Cytokine, 2015. **76**(1): p. 25-37.
104. Li, N., et al., *Alarmin function of cathelicidin antimicrobial peptide LL37 through IL-36gamma induction in human epidermal keratinocytes*. J Immunol, 2014. **193**(10): p. 5140-8.
105. Schlitzer, A., et al., *IRF4 transcription factor-dependent CD11b+ dendritic cells in human and mouse control mucosal IL-17 cytokine responses*. Immunity, 2013. **38**(5): p. 970-83.
106. Buonocore, S., et al., *Innate lymphoid cells drive interleukin-23-dependent innate intestinal pathology*. Nature, 2010. **464**(7293): p. 1371-5.
107. Morrison, P.J., S.J. Ballantyne, and M.C. Kullberg, *Interleukin-23 and T helper 17-type responses in intestinal inflammation: from cytokines to T-cell plasticity*. Immunology, 2011. **133**(4): p. 397-408.
108. Awasthi, A., et al., *Cutting edge: IL-23 receptor gfp reporter mice reveal distinct populations of IL-17-producing cells*. J Immunol, 2009. **182**(10): p. 5904-8.
109. Zenewicz, L.A. and R.A. Flavell, *Recent advances in IL-22 biology*. Int Immunol, 2011. **23**(3): p. 159-63.
110. Vivier, E., H. Spits, and T. Cupedo, *Interleukin-22-producing innate immune cells: new players in mucosal immunity and tissue repair?* Nat Rev Immunol, 2009. **9**(4): p. 229-34.
111. Colonna, M., *Interleukin-22-producing natural killer cells and lymphoid tissue inducer-like cells in mucosal immunity*. Immunity, 2009. **31**(1): p. 15-23.
112. Chen, F., et al., *mTOR Mediates IL-23 Induction of Neutrophil IL-17 and IL-22 Production*. J Immunol, 2016. **196**(10): p. 4390-9.
113. Aden, K., et al., *Epithelial IL-23R Signaling Licenses Protective IL-22 Responses in Intestinal Inflammation*. Cell Rep, 2016. **16**(8): p. 2208-2218.
114. Parks, O.B., et al., *Interleukin-22 Signaling in the Regulation of Intestinal Health and Disease*. Front Cell Dev Biol, 2015. **3**: p. 85.
115. Mizoguchi, A., *Healing of intestinal inflammation by IL-22*. Inflamm Bowel Dis, 2012. **18**(9): p. 1777-84.
116. Becker, C., et al., *Cutting edge: IL-23 cross-regulates IL-12 production in T cell-dependent experimental colitis*. J Immunol, 2006. **177**(5): p. 2760-4.
117. Mahil, S.K., et al., *An analysis of IL-36 signature genes and individuals with IL1RL2 knockout mutations validates IL-36 as a psoriasis therapeutic target*. Sci Transl Med, 2017. **9**(411).
118. Dietrich, D. and C. Gabay, *Inflammation: IL-36 has proinflammatory effects in skin but not in joints*. Nat Rev Rheumatol, 2014. **10**(11): p. 639-40.

119. Gresnigt, M.S. and F.L. van de Veerdonk, *Biology of IL-36 cytokines and their role in disease*. Semin Immunol, 2013. **25**(6): p. 458-65.
120. Deepak, P. and W.J. Sandborn, *Ustekinumab and Anti-Interleukin-23 Agents in Crohn's Disease*. Gastroenterol Clin North Am, 2017. **46**(3): p. 603-626.
121. Bamias, G., C.A. Dinarello, and J. Rivera-Nieves, *Innate cytokines dictate the fate of acute intestinal inflammation*. Gastroenterology, 2015. **148**(1): p. 248-50.
122. Bersudsky, M., et al., *Non-redundant properties of IL-1alpha and IL-1beta during acute colon inflammation in mice*. Gut, 2014. **63**(4): p. 598-609.
123. Elinav, E., et al., *NLRP6 inflammasome regulates colonic microbial ecology and risk for colitis*. Cell, 2011. **145**(5): p. 745-57.
124. Seregin, S.S., et al., *NLRP6 function in inflammatory monocytes reduces susceptibility to chemically induced intestinal injury*. Mucosal Immunol, 2017. **10**(2): p. 434-445.
125. Monticelli, L.A., et al., *IL-33 promotes an innate immune pathway of intestinal tissue protection dependent on amphiregulin-EGFR interactions*. Proc Natl Acad Sci U S A, 2015. **112**(34): p. 10762-7.
126. Dinarello, C.A., et al., *Suppression of innate inflammation and immunity by interleukin-37*. Eur J Immunol, 2016. **46**(5): p. 1067-81.
127. McNamee, E.N., et al., *Interleukin 37 expression protects mice from colitis*. Proc Natl Acad Sci U S A, 2011. **108**(40): p. 16711-6.
128. Mullineaux-Sanders, C., et al., *Citrobacter rodentium-host-microbiota interactions: immunity, bioenergetics and metabolism*. Nat Rev Microbiol, 2019. **17**(11): p. 701-715.
129. Silberger, D.J., C.L. Zindl, and C.T. Weaver, *Citrobacter rodentium: a model enteropathogen for understanding the interplay of innate and adaptive components of type 3 immunity*. Mucosal Immunol, 2017. **10**(5): p. 1108-1117.
130. Collins, J.W., et al., *Citrobacter rodentium: infection, inflammation and the microbiota*. Nat Rev Microbiol, 2014. **12**(9): p. 612-23.
131. Basu, R., et al., *Th22 cells are an important source of IL-22 for host protection against enteropathogenic bacteria*. Immunity, 2012. **37**(6): p. 1061-75.
132. Abt, M.C., et al., *Innate Immune Defenses Mediated by Two ILC Subsets Are Critical for Protection against Acute Clostridium difficile Infection*. Cell Host Microbe, 2015. **18**(1): p. 27-37.
133. Guo, X., et al., *Innate Lymphoid Cells Control Early Colonization Resistance against Intestinal Pathogens through ID2-Dependent Regulation of the Microbiota*. Immunity, 2015. **42**(4): p. 731-43.
134. Plank, M.W., et al., *Th22 Cells Form a Distinct Th Lineage from Th17 Cells In Vitro with Unique Transcriptional Properties and Tbet-Dependent Th1 Plasticity*. J Immunol, 2017. **198**(5): p. 2182-2190.
135. Vallance, B.A., et al., *Mice lacking T and B lymphocytes develop transient colitis and crypt hyperplasia yet suffer impaired bacterial clearance during Citrobacter rodentium infection*. Infect Immun, 2002. **70**(4): p. 2070-81.
136. Aychek, T., et al., *IL-23-mediated mononuclear phagocyte crosstalk protects mice from Citrobacter rodentium-induced colon immunopathology*. Nat Commun, 2015. **6**: p. 6525.
137. Dann, S.M., et al., *IL-6-dependent mucosal protection prevents establishment of a microbial niche for attaching/effacing lesion-forming enteric bacterial pathogens*. J Immunol, 2008. **180**(10): p. 6816-26.

138. Tsai, P.Y., et al., *IL-22 Upregulates Epithelial Claudin-2 to Drive Diarrhea and Enteric Pathogen Clearance*. Cell Host Microbe, 2017. **21**(6): p. 671-681 e4.
139. Lebeis, S.L., et al., *Interleukin-1 receptor signaling protects mice from lethal intestinal damage caused by the attaching and effacing pathogen *Citrobacter rodentium**. Infect Immun, 2009. **77**(2): p. 604-14.
140. Kovach, M.A., et al., *IL-36gamma is a crucial proximal component of protective type-1-mediated lung mucosal immunity in Gram-positive and -negative bacterial pneumonia*. Mucosal Immunol, 2017. **10**(5): p. 1320-1334.
141. Nanjo, Y., et al., *Overlapping Roles for Interleukin-36 Cytokines in Protective Host Defense against Murine *Legionella pneumophila* Pneumonia*. Infect Immun, 2019. **87**(1).
142. Verma, A.H., et al., *IL-36 and IL-1/IL-17 Drive Immunity to Oral Candidiasis via Parallel Mechanisms*. J Immunol, 2018. **201**(2): p. 627-634.
143. Brahmakshatriya, V., et al., *IL-6 Production by TLR-Activated APC Broadly Enhances Aged Cognate CD4 Helper and B Cell Antibody Responses In Vivo*. J Immunol, 2017. **198**(7): p. 2819-2833.
144. Heink, S., et al., *Trans-presentation of IL-6 by dendritic cells is required for the priming of pathogenic TH17 cells*. Nat Immunol, 2017. **18**(1): p. 74-85.
145. Xu, D., R. Mu, and X. Wei, *The Roles of IL-1 Family Cytokines in the Pathogenesis of Systemic Sclerosis*. Front Immunol, 2019. **10**: p. 2025.
146. Ahlfors, H., et al., *IL-22 fate reporter reveals origin and control of IL-22 production in homeostasis and infection*. J Immunol, 2014. **193**(9): p. 4602-13.